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THE UNIVERSITY OF ALBERTA

THE MOLECULAR RADIOBIOLOGY OF NUCLEIC ACIDS

by

(C) ALFRED FRANK FUCIARELLI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND

RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES

(RADIOLOGY AND DIAGNOSTIC IMAGING)

EDMONTON, ALBERTA

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DEDICATION

To those who fill my life with joy and happiness

- my family and friends

Attempting to escape one's destiny
is an exercise in futility.

-Alfred F. Fuciarelli

ABSTRACT

In addition to radiolytic adenine release, radiolysis of adenosine 5'-monphosphate, in the absence of oxygen, can result in the formation of 8-hydroxyadenosine 5'-monophosphate and both the (R)- and (S)-epimer of 8,5'-cycloadenosine 5'-monophosphate. The mononucleoside derivatives of these modified nucleotides were also observed in irradiated solutions of adenosine and in the enzyme hydrolysates of irradiated solutions of polyadenylic acid (poly A) using high-performance liquid chromatography (HPLC).

In an effort to detect 8,5'-cyclo(deoxy)adenosine formation in irradiated nucleic acids, polyclonal antiserum were raised with specificity to the 8,5'-cycloadenosine 5'-monophosphate moiety and used in an enzyme-linked immunosorbent assay (ELISA). The 8,5'-cyclo(deoxy)adenosine moiety could be detected in nitrous oxide-saturated aqueous solutions containing unhydrolyzed poly A at 10 Gy and DNA at 200 Gy using the colorimetric ELISA. Correlation of product yield measured by ELISA with HPLC analysis of irradiated, enzyme-hydrolyzed solutions of poly A revealed that the ELISA was precisely reflecting changes in the combined yield of (R)- and (S)-8,5'-cycloadenosine.

Radiation chemical experiments, including studies on the pH- and oxygen-dependencies for product formation have revealed that 8,5'-cyclo(deoxy)nucleoside formation may serve as an unequivocal probe of an initial hydroxyl radical-induced event from the (deoxy)adenosine moiety in irradiated nucleic acids. The ELISA

tecnique was used to assess product yield in irradiated solutions of poly A under conditions designed to test 8,5'-cycloadenosine formation as a possible probe of radiation chemical events. In this context, 8,5'-cycloadenosine formation was inhibited by the addition of hydroxyl radical scavengers, in the presence of molecular oxygen or in the presence of a series of nitroaromatic radiosensitizers, the latter in a way which generally increased with increasing electron affinity.

Finally, an ultrasensitive modification of the conventional ELISA permitted detection of 8,5'-cyclo(deoxy)adenosine moieties in DNA irradiated in solution with twenty-fold greater sensitivity. this increased sensitivity was insufficient to permit product detection in DNA extracted from oxygen- and thiol-depleted, irradiated V79 fibroblasts.

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ALFRED F. FUCIARELLI

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CHAPTER 1

THE MOLECULAR RADIOBIOLOGY OF NUCLEIC ACIDS

A. INTRODUCTION

Physical and chemical processes occurring in the order of picoseconds to milliseconds following energy deposition by ionizing radiation are ultimately responsible for phenomona such as mutagenesis, carcinogenesis and proliferative cell death which occur over the longer term (1-3). In the radiochemical stage, waves of reactive radicals are generated largely from water radiolysis; each wave being less reactive and each chemical reaction being more specific than its predecessor (4). The most important event to occur at this stage may be the interaction of a primary radical with a cellular molecule to produce an intermediate secondary radical on the biomolecule. The consequence of this interaction to a living cell is dependent upon the position that the modified biomolecule occupies in the hierarchy of critical biomolecules ultimately responsible for the functional integrity of living cells. Evidence reviewed by Chapman and Gillespie (3) suggests that deoxyribonucleic acid (DNA) is at the apex of this hierarchy. Characterizing and examining the fate of these intermediate radicals has been of considerable interest in cellular radiobiology.

The radical competition model provides a conceptual framework for studies involving radiosensitization by oxygen or electron affinic radiosensitizers, and radioprotection associated with

hydrogen-donating compounds (2,3,5-14). Although modification of the secondary radical is a proposed mechanism for such changes observed at the cellular level, rigorous testing of this hypothesis at the biochemical level using radiation-induced molecular products as probes of the radical competition model has not been done. The aim of this thesis was to develop the use of specific molecular products as "probes" of radical events underlying the radical competition model. This was accomplished by performing experiments designed to better understand the mechanism leading to radiation-induced product formation, developing sensitive immunochemical assays for detection of molecular products in irradiated DNA and testing specific molecular products for their potential use as "probes" of radical events.

B. WATER RADIOLYSIS: PRIMARY RADICALS AND THEIR RELATIVE IMPORTANCE IN RADIOBIOLOGY

Radiation-induced chemical alterations in the structure of critical biomolecules, and the biochemical effects resulting from these events in the living organism are largely related to water radiolysis. A typical mammalian cell consists of water (70%), proteins (18%), DNA (0.25%), RNA (1.1%), lipids (5%), carbohydrates (2%), metabolites and inorganic ions (3.65%) in a total volume of 4×10^{-12} ml (15). Due to the high water content of cells, a significant extent of radiation damage results primarily from the indirect effect; that is, the absorption of energy by a molecule such as water, leading

to the formation of reactive intermediates which, can in turn, interact with critical molecular targets. Alternatively, direct effects involving the direct ionization or excitation of critical molecular targets could account for up to 30-40% of mammalian cell inactivation (11,16,17).

The decomposition of water by low-linear energy transfer radiations [<0.56 fJ/μm (3.5 keV/μm)] has been extensively studied and reviewed (18,19). Incident photons set in motion photo- and Compton- electrons which initially interact with target molecules to produce ionizations, super excitations and excitations within 10^{-17} - 10^{-15} seconds (1,3). Absorption of energy is random (20-22), with energy deposition occurring on the average of 100-500 nm apart in 3.2 - 16 aJ (20 - 100 eV) packets. In the case of water radiolysis (equation 1),

$$H_2O \rightsquigarrow e^-_{aq},\ H^{\cdot},\ {}^{\cdot}OH,\ H_3O^+,\ H_2,\ H_2O_2 \qquad (1)$$

excited and ionized water molecules undergo rapid reactions which result in several radical ($^{\cdot}OH$, e^-_{aq}, $^{\cdot}H$) and molecular (H_2, H_2O_2) species (Table 1) over a time scale of 10^{-14} to 10^{-8} seconds within a localized volume of high concentration termed a "spur" (23). The proposed mechanisms of formation of these radical and molecular species and their steady-state yields at neutral pH at 10^{-8} seconds is given in terms of their G values (viz., molecules of product produced per 16 aJ (100 eV) absorbed) in Table 1.

TABLE 1: ACTION OF IONIZING RADIATIONS ON WATER[1]

$H_2O \rightsquigarrow e^-_{aq}, H^\cdot, {}^\cdot OH, H_3O^+, H_2, H_2O_2$

Radical products

$$H_2O \longrightarrow H_2O^+ + e^-$$

$$e^- + nH_2O \longrightarrow e^-_{aq}$$

$$H_2O^+ + H_2O \longrightarrow {}^\cdot OH + H_3O^+$$

$$H_2O \longrightarrow H_2O^* \longrightarrow H^\cdot + {}^\cdot OH$$

$$e^-_{aq} + H_3O^+ \longrightarrow H^\cdot + H_2O$$

Molecular products

$$H^\cdot + H^\cdot \longrightarrow H_2$$

$$e^-_{aq} + e^-_{aq} \xrightarrow{2H_2O} H_2 + 2OH^-$$

$$e^-_{aq} + H^\cdot \xrightarrow{H_2O} H_2 + OH^-$$

$${}^\cdot OH + {}^\cdot OH \longrightarrow H_2O_2$$

Yields

Species:	e^-_{aq}	$H^\cdot$	${}^\cdot OH$	H_3O^+	H_2	H_2O_2
G value[2]	2.7	0.55	2.7	2.7	0.45	0.7

1) Refers to low-linear energy transfer radiations [<0.56 fJ/μm (24)].

2) G = molecules per 100 eV (1 eV = 0.16 aJ)

As a result of water radiolysis, highly reactive oxidizing ($^{\cdot}OH$, H_2O_2) and reducing (e^-_{aq}, $^{\cdot}H$) species are formed (18). Studies in cellular radiobiology using alcohols (17), sulfhydryls (17) and dimethyl sulfoxide (11,25) to scavenge hydroxyl radicals, suggest that hydroxyl radicals are responsible for 60-70% of cell inactivation and that the contribution of e^-_{aq} and $^{\cdot}H$ to inactivation is negligible. However, Chapman and Gillespie, (3) point out that cell inactivation resulting from e^-_{aq} or other reducing species is difficult to study because of quick transfer of target radical anions, produced from e^-_{aq} attack, to more electron affinic molecules in the cell such as oxygen. Although the yield of e^-_{aq} is approximately that of the hydroxyl radical (Table 1), the efficiency of inactivation for the reaction of hydrated electrons with DNA is only 8 per cent (half that of hydroxyl radicals (26)). In addition, the reaction rate constant of hydrated electrons with DNA is three times smaller than that of hydroxyl radicals (27). In the case of hydrogen atoms, which have a steady-state yield of only twenty percent of that of $^{\cdot}OH$ or e^-_{aq}, the contribution to cell inactivation is not known. Michaels and Hunt (28) suggest that a significant component of the indirect effect may be attributed to hydrogen atoms.

In the presence of molecular oxygen, the hydrated electron (e^-_{aq}) and the hydrogen atom ($H^{\cdot}$) will react to form the superoxide radical anion ($O_2^{\cdot-}$) and its conjugate acid, the hydroperoxyl radical ($HO_2^{\cdot}$) (equation 2).

$$e^-_{aq} + O_2 \longrightarrow O_2^{\cdot-} \overset{H^+}{\rightleftharpoons} HO^{\cdot}_2 \longleftarrow O_2^{\cdot-} + H^+ \qquad (2)$$

Neither $O_2^{\cdot-}$ nor $HO_2^{\cdot}$ are as effective in cell inactivation as the hydroxyl radical. The $HO_2^{\cdot}$ radical is produced at low yield at physiological pH and the $O_2^{\cdot-}$ radical may undergo either dismutation or enzyme reduction with superoxide dismutase to form hydrogen peroxide which is subsequently converted to water and oxygen by catalases and peroxidases (29).

The hydroxyl radical may therefore be the predominant species resulting from water radiolysis which is capable of interacting with critical target molecules in cellular systems. The rate of reaction of hydroxyl radicals with critical targets for proliferative cell death was 9×10^8 seconds^{-1} (30). The average distance of migration of the hydroxyl radical is approximately 4 nm from the site of formation with an average lifetime of 10^{-9} seconds (3). Thus a significant proportion of the hydroxyl radical-induced damage to critical targets must occur within this distance. Chapman and Gillespie (3) have extensively reviewed the evidence which suggests that nuclear DNA is the most sensitive molecular species within a mammalian cell leading to loss of proliferative capacity. Since the average lateral packing diameter of double-stranded B-DNA is 1.93 nanometers (31), approximately one half the average distance of migration of the hydroxyl radical, the site of the initial spur must be close to, or within, the hydration layer of the nuclear DNA to produce a critical lesion.

The interaction of hydroxyl radicals with biological molecules results in the formation of secondary radicals primarily through hydrogen abstraction from a carbon atom or addition across sites of unsaturation (18,32). In general, distribution of hydroxyl radical attack is dependent upon the presence or absence of unsaturation, the number and strengths of C-H bonds and, because of the electrophilic nature of the hydroxyl radical, the presence or absence of charged groups in the molecule (33,34). In the case of nucleic acids the base moiety can undergo redox reactions or addition reactions with free radicals; the aliphatic sugar moiety may only undergo hydrogen atom abstraction reactions. Reactions of hydroxyl radicals with either the base or sugar moieties can be influenced by the charged state of either the base or phosphate group (35).

The relative importance of hydroxyl radical-induced events may be stressed by reference to studies that have used dimethyl sulfoxide to scavenge the yield of hydroxyl radicals to achieve radioprotection and studies that have used nitrous oxide to increase the yield of hydroxyl radicals, the latter condition leading to radiosensitization in many systems.

Scavenging of hydroxyl radicals can lead to radioprotection (17,36,37). Dimethyl sulfoxide (DMSO) is an efficient $^{\cdot}OH$ scavenger which results in the conversion of the $^{\cdot}OH$ radical to the much less reactive methyl radical ($CH_3^{\cdot}$) which, in the presence of oxygen, reacts by addition to form the methylperoxy radical ($CH_3OO^{\cdot}$) (38,39) (equation 3)

$$CH_3\text{-}\overset{\overset{O}{\|}}{S}\text{-}CH_3 + {}^{\cdot}OH \longrightarrow [CH_3\text{-}\overset{\overset{O}{|}}{\underset{\underset{OH}{|}}{S}}\text{-}CH_3]^{\cdot} \longrightarrow {}^{\cdot}CH_3 + CH_3\text{-}\overset{\overset{O}{\|}}{S}\text{-}OH \quad (3)$$

$$k = 5.8 \times 10^9\ dm^3 \cdot mol^{-1} \cdot s^{-1}$$

Dimethyl sulfoxide has been shown to be an effective radioprotector in cellular systems (11,25,38,40-44) and in cells irradiated with heavy charged-particle beams (45).

Potentiation of radiation damage might be anticipated if the initial yield of primary radicals is increased. The yield of the hydroxyl radical can be doubled relative to its yield in nitrogen-saturated aqueous solution by performing the irradiation in the presence of nitrous oxide (N_2O) wherein the hydrated electron can be converted to the hydroxyl radical [Equation 4, (46)].

$$e^-_{aq} + N_2O \xrightarrow{H_2O} N_2 + {}^{\cdot}OH + OH^- \quad (4)$$

$$k = 5.6 \times 10^9\ dm^3 \cdot mol^{-1} \cdot s^{-1}$$

The hydrated electron (e^-_{aq}) was reported to have a steady state G value of 2.7 (±0.1) based on direct measurement of the e^-_{aq} absorption approximately one microsecond after irradiation using pulse radiolysis techniques (47). In the presence of nitrous oxide, assuming total conversion ($e^-_{aq} \longrightarrow {}^{\cdot}OH$), the yield of the hydroxyl radical should approach G = 5.4. This assumption has been used to

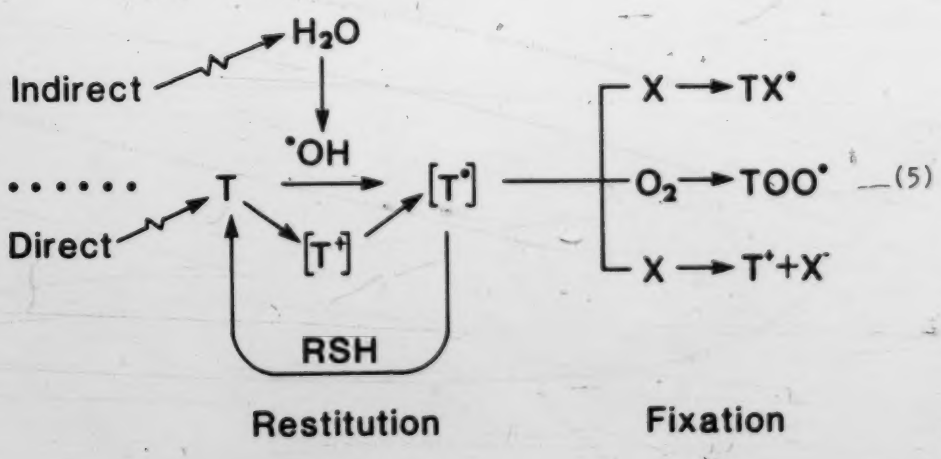
explain the nitrous oxide-induced sensitization of irradiated bacterial spores (48,49) and bacteriophage (50). However, it should be noted that increased radiosensitization with nitrous oxide has not been observed in Micrococcus sodonenis (51) nor in any mammalian cell system to date except in the inexplicable case where cells were bathed in medium containing 3 $mol \cdot dm^{-3}$ dimethyl sulfoxide (44). Therefore, while the radiation chemistry of nitrous oxide-saturated solutions may be well understood, radiosensitization of cells using this technique is not successful.

In the light of this discussion which focused upon primary radical formation and the deleterious consequences of radicals in cellular systems, the hydroxyl radical is considered to be the predominant species attacking critical cellular targets to produce secondary radicals. The fate of these secondary radicals shall be discussed in the context of the radical competition model.

C. THE RADICAL COMPETITION MODEL: INTERMEDIATE RADICALS IN MOLECULAR RADIOBIOLOGY

The reactions of primary radicals originating from water radiolysis can produce secondary radicals on cellular molecules which may react further in the cellular milieu. I shall adopt the term "intermediate radical" to refer to a radical centered on a critical cellular molecule. The fate of these intermediate radicals has received much attention in radiation research. A number of possible competing radical reactions, and descriptions of the

competition process, has resulted in the formulation of a hypothesis amenable to experimental testing at the cellular and molecular level. The radical competition model is based on studies in polymer radiation chemistry (5) and has been used by others as a conceptual framework to study the oxygen effect, radiosensitization by electron affinic compounds, and radioprotection by thiol-depleting agents. Excellent reviews are available which provide additional details on the model than shall be presented in this brief discussion (2,9,13). In the context of the model, oxidizing radicals (i.e., $^{\bullet}OH$) are damaging because they attack critical targets (T) in the cell (DNA being the most important target molecule) to produce intermediate radicals ($T^{\bullet}$). As previously suggested, 60-70% of mammalian cell inactivation can be attributed to oxidizing species; primarily by hydroxyl radical-induced addition and abstraction reactions, and the remaining 30-40% is attributed to direct effects (11,17). Both indirect and direct effects can lead to intermediate radicals ($T^{\bullet}$) (Equation 5).



(5)

Chemical competition for the intermediate radical (T·) occurring over the timescale of 10^{-6} to 10^{-3} seconds (2,3) has been suggested as the basis for the observed radiobiological response at the molecular and cellular level. Fixation of the radical by oxidative processes may occur by either the addition of molecular oxygen (6,7,52-54) or other electron affinic compounds (9,11,55) to the intermediate radical (T·). Fixation of damage results in chemically distinct changes to the target molecules; changes that might be amenable to enzymatic repair over a much longer timescale. If not repaired these molecular changes may lead to changes at the cellular level including mutagenesis and proliferative death. An alternative reaction, in competition for the intermediate radical (T·), is a fast chemical repair process involving hydrogen atom donation from compounds such as cellular nonprotein sulfhydryls (thiols) [e.g. glutathione, L-cysteine, cysteamine, coenzyme A, and dipeptides (56)], which results in reconstitution of the original target molecule and radioprotection at the molecular and cellular level (5-14).

Evidence in favor of the radical competition model comes from work involving depletion of intracellular nonprotein thiols in mammalian cells. Depletion of endogenous thiols leads to increased effectiveness of an exogenous radiation sensitizer while not appreciably affecting the response of treated cells in the absence of sensitizer, an effect observed only for hypoxic cells and no effects of thiol depletion or radiosensitizer addition were noted for aerobic cells (57-59). Bump et al. (60) demonstrated a ten-fold

increase in the radiosensitizing efficiency of misonidazole in hypoxic cells depleted (> 90%) of endogenous glutathione. As a follow up experiment, Koch et al. (61) demonstrated that glutathione depletion resulted in a 7-fold decrease in the K_m (concentration for half-maximal response) for radiosensitization by misonidazole, and only a 2.5-fold decrease in the K_m for radiosensitization with oxygen. The difference in the enhanced sensitivity between oxygen and misonidazole was interpreted by Koch et al. (61) to mean that the simplest form of the radical competition model may not accurately model the competition between oxygen fixation and chemical repair when the data are rigorously scrutinized. In related studies van der Schans et al. (14) measured the effects of intracellular glutathione depletion on the expression of radiation-induced cell death and correlated this with the induction of radiation-induced single- and double-strand breaks in DNA. van der Schans et al. (14) suggest that the data "fit" a simple competition model involving a competition between sensitizers and protectors with intermediate DNA radicals. In the oxygen concentration range 0.1 - 10 $\mu mol \cdot dm^{-3}$, 90% sensitization for single- and double-strand breaks and 50% sensitization for cell killing were observed (14). The discrepancy in the observed sensitization for DNA strand breaks and for cell killing is related to the extent to which the critical secondary radicals participate in the oxygen-thiol competition. For example in DNA, sugar type radicals participate in this competition, yet base radicals do not (62). Therefore, much evidence has accumulated

to suggest that intracellular thiols compete with oxygen and electron affinic radiosensitizers with respect to radiation-induced damage to prevent damage fixation.

Fixation of damage by the reaction of molecular oxygen with transforming DNA radicals proceeds 200-fold faster than the competing repair reaction by hydrogen donation from dithiothreitol at equal concentrations of oxygen and dithiothreitol (63). This ratio is in good agreement with the ratio of 160 obtained in yeast (64) and 200-300 for Escherichia coli (36). On the basis of this 200-fold ratio of reaction rates, at least 250 $mmol \cdot dm^{-3}$ dithiothreitol would be required to compete efficiently with ~100 per cent oxygen (1.3 $mmol \cdot dm^{-3}$) for the DNA radicals (63). The 200-fold ratio of reaction rates between oxygen fixation and chemical repair is an important concept to bear in mind when attempting to test predictions of the radical competition model.

The critical target sites of radiation-induced damage involved in the competition between reductive protection by sulfhydryls and oxidative sensitization by oxygen or electron affinic radiosensitizers at the cellular level have not been elucidated. However, cell membranes, particularly membrane-DNA complexes (65,66) DNA double-strand breaks (67-73) and free radical sites on, or associated with, nuclear DNA (2) have all been implicated. The interaction of sulfhydryl compounds with radiation-induced free radical sites on DNA or its constituents leading to reconstitution has been demonstrated for nucleotides (62,74,75), polynucleotides (76-78)

and DNA (63,79-81). At the molecular level, the radical competition model can be easily tested if the fate of any one of these specific radicals can be followed as a function of sensitization and protection conditions.

D. TESTING THE RADICAL COMPETITION MODEL BY MONITORING THE FATE OF SPECIFIC MOLECULAR PRODUCTS WHICH SERVE AS UNEQUIVOCAL PROBES OF INTERMEDIATE RADICALS ON NUCLEIC ACIDS

The radical competition model is based on the presumption that the radical produced on a critical biomolecule undergoes antagonistic competition with sensitizing (i.e. oxygen, electron affinic radiosensitizers) and protecting (i.e. sulfhydryl compounds) species. Conceptually, the intermediate radical may be used as a "probe" of the radical competition model provided that a steady-state product can be formed and assayed using a technique with sufficient sensitivity and specificity. With respect to the radiation chemistry of nucleic acids and their constituents, a wealth of knowledge has been accumulated and reviewed (32,82-86). Despite this literature, few radiation-induced lesions offer unequivocal[1] records of the initial radical event which led to their formation. As reviewed by Ward (87), DNA strand breaks do not serve as an appropriate "probe" for the radical competition model because the measured yield reflects the recruitment of breaks resulting from damage which results in loss of a base, leaving an alkaline labile apurinic or apyrimidinic site or from the production of a base damaged product whose glycosylic

1. Unequivocal refers to the ability to establish the initial site of radical attack in a molecule based on the final radiation-induced modification.

bond was easily hydrolyzed in alkali. Radiation-induced base release is also an inappropriate probe for the radical model since release of the base can occur from radical events at a number of centers in the molecule. Most radiation-induced molecular lesions involving modifications in the sugar and/or base moieties are inappropriate as probes of the radical model due to an incomplete understanding of the mechanism of formation, or low product yield. However, 8,5'-cyclonucleotide(side)[2] formation has been established as an unequivocal record of an initial radical event which occurred on a nucleic acid constituent as a result of the work presented in this thesis and might therefore serve as a steady-state probe of an intermediate radical centered at C(5') of the sugar moiety to test predictions of the radical competition model.

Formation of 8,5'-cyclonucleotides(sides) from aqueous solutions of purine containing nucleic acid constituents irradiated in the absence of oxygen represents a novel radiolysis product. Keck (88) demonstrated that an intramolecular cyclization, resulting in the formation of a C(8)-C(5') bond, occurred in nitrogen-saturated solutions of the 5'-monophosphates of adenosine, guanosine, inosine and deoxyadenosine using proton nuclear magnetic resonance techniques. The mechanism of formation of the 8,5'-cyclonucleoside(tide) (Figure 1):

2. The notation 8,5'-cyclonucleotide(side) is used to refer to either the nucleotide or nucleoside derivatives.

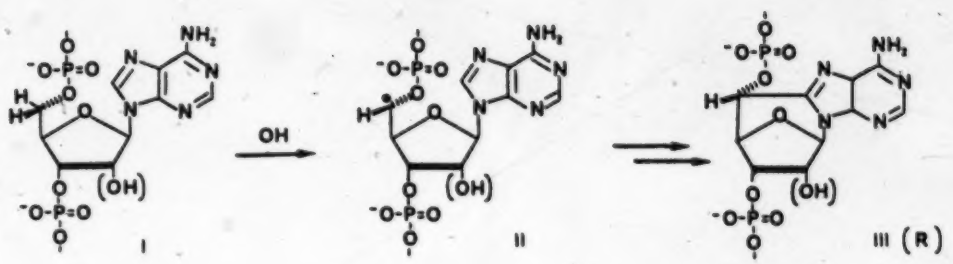


Figure 1: Mechanism of formation of 8,5'-cycloadenosine

involves hydrogen atom abstraction from the C(5') carbon of the (deoxy)ribose ring by a hydroxyl radical followed by addition of the C(5') radical to the C(8) position of the purine base (88). Electron spin resonance studies have provided evidence for the existence of C(5') radicals in solutions of irradiated nucleotides and nucleosides (89-93), and the nucleophilic addition of alkyl radicals to C(8) of purine bases, a center which has proven to be highly reactive (94), is a well-known phenomenon (95-98). The N(7)-centered radical is neutralized by a one electron oxidation, the least understood step in the reaction mechanism, which results in 8,5'-cyclonucleoside(tide) formation. In addition to the work of Keck (88), formation of 8,5'-cyclonucleotides(sides) has been reported in deoxygenated, irradiated solutions of deoxyadenosine (99), deoxyguanosine (100), adenosine (101), adenosine 5'-monophosphate (35,102,103), polyadenylic acid (101,104,105) and DNA (100,104). To date, formation of 8,5'-cyclodeoxynucleosides

has yet to be reported in DNA extracted from irradiated cells. Formation of both the (R)- and (S)-epimers at the C(5') position of 8,5'-cyclonucleotides(sides) was first demonstrated in monomers by Raleigh and Fuciarelli (35) and in nucleic acids (105) for adenine-containing compounds. Similar observations have been reported for the formation of (R)- and (S)-8,5'-cyclodeoxyguanosine in irradiated solutions of deoxyguanosine and DNA (100). The factors directing stereoselective formation of 8,5'-cyclonucleotides(sides) in irradiated monomers and polymers are discussed in Chapter 8.

Formation of 8,5'-cyclonucleotides(sides) is completely inhibited in aerated solutions (88,103,104,Chapter 4). In mononucleotides, maximum yield occurs in nitrous oxide, as compared to nitrogen-saturated solution (103,104) and complete inhibition of product formation occurs at a liquid-phase oxygen concentration of 1.6% pO_2 with a K_m of 0.08% pO_2 (Chapter 4).

The biochemical implications of 8,5'-cyclodeoxynucleoside formation on the fidelity of information transfer by DNA are perhaps more subtle than elimination of the base or overt strand breaks. The ability and efficiency by which the repair enzymes operate becomes a major factor in the consequences of any type of chemical alteration to the DNA structure. The substrate specificity of various enzymes with 8,5'-cycloAMP epimers may have implications with regard to the efficiency by which cellular enzymes might possibly repair this type of lesion if it is formed in irradiated cells. Raleigh and Blackburn (106) were the first authors to correctly report that

snake venom 5'-nucleotidase, which completely hydrolyzes 5'-AMP to adenosine, does not hydrolyze the (S)-epimer of 8,5'-cycloAMP, the identity of which was established by X-ray crystallography (107). On the other hand, the (R)-epimer is hydrolyzed by 5'-nucleotidease (106,108). The (R)-epimer has proven to be a substrate for pig muscle adenyl kinase and the resulting 8,5'-cycloadenosine diphosphate, a substrate for rabbit muscle pyruvate kinase (107). However, the (S)-epimer is not a substrate for pig muscle adenyl kinase (107). Interestingly, it is the (R)-epimer which, due to conformational constraints (see Chapter 8), predominates in yield over the (S)-epimer in irradiated nucleic acids (100,105). These data raise the possibility that DNA repair enzymes may selectively hydrolyze one epimer of 8,5'-cyclodeoxyadenosine and not the other, thus leaving an unrepaired molecular lesion within the DNA molecule.

In this thesis, the radiation chemistry of adenine-containing monomers (Chapters 2 and 4) and polymers (Chapters 5 and 7) is studied. These studies reveal that 8,5'-cyclo(deoxy)nucleotide(side) formation is an unequivocal, steady-state probe of an initial hydroxyl radical-induced event on a nucleic acid constituent. Using polyclonal antiserum raised with specificity to the 8,5'-cycloadenosine moiety in an enzyme-linked immunosorbent assay (ELISA), formation of 8,5'-cyclo(deoxy)adenosine was demonstrated in irradiated nucleic acids (Chapter 6). Formation of the 8,5'-cyclonucleoside was assessed in the presence of hydroxyl radical scavengers and nitroaromatic radiosensitizers (Chapters 6 and 9). Finally, an ultrasensitive

modification to the basic ELISA was used in an attempt to detect 8,5'-cyclodeoxynucleoside formation in DNA extracted from irradiated, deoxygenated, thiol-depleted V79 fibroblasts (Chapter 10).

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CHAPTER 2

DISTRIBUTION OF DAMAGE IN IRRADIATED 5'-AMP: 8,5'-CYCLOAMP, 8-HYDROXYAMP AND ADENINE RELEASE

INTRODUCTION

The yields of 8,5'-cycloadenosine 5'-monophosphate (8,5'-cycloAMP) formation and phosphate release in solutions of adenosine 5'-monophosphate (5'-AMP) irradiated in the absence of oxygen were shown to have a strong pH dependence (1). It was suggested that this could be due to a change in the rate of hydroxyl radical attack at sugar and base moieties in response to pH-induced changes in the site of protonation in 5'-AMP (1). Evidence that such changes in $^{\cdot}OH$ attack does occur has now emerged from a study of the acid-stable radiolysis products of 5'-AMP, (S)-8,5'-cycloAMP (I), (R)-8,5'-cycloAMP (II) and 8-hydroxyAMP (III), and radiolytic adenine release. It has been found that the yields of base damage (8-hydroxyAMP) and sugar damage (8,5'-cycloAMP and adenine release) show pH dependencies which can be correlated with the acid-base equilibria of 5'-AMP. In addition, it has been found that the relative yields of the (R)- and (S)-epimers of 8,5'-cycloAMP are pH-dependent with the (S)-epimer predominating at high pH and the (R)-epimer predominating at low pH. Finally, ionization of the adenine base

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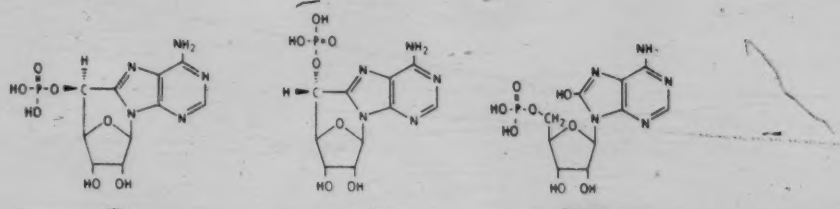
at low pH is accompanied by a decrease in $^{\bullet}OH$ attack away from adenine. This is consistent with increases in phosphate release, 8,5'-cycloAMP formation and adenine release.



(I) (II) (III)

MATERIALS AND METHODS

Adenosine 5'-monophosphate and 8-bromoadenosine 5'-monophosphate were purchased from the Sigma Chemical Co. and used as received. The 8-hydroxyadenosine 5'-monophosphate was synthesized according to a published procedure (2) and characterized by infrared and ultraviolet spectroscopy and fast atom bombardment mass spectrometry. Other chemicals were reagent grade quality and were used as received.

Irradiations were carried out with a Gammacell 220 ^{60}Co gamma radiation source (Atomic Energy of Canada Ltd.) at a dose-rate of 79 Gy/min as measured by Fricke dosimetry ($G(Fe^{3+}) = 15.6$). For irradiations on a preparative scale 18.0 g of 5'-AMP were dissolved in 500 ml (104 $mmol \cdot dm^{-3}$) of doubly distilled water adjusted to pH 9 with sodium hydroxide. The solution was bubbled with nitrous oxide for fifteen minutes before and then throughout the time of irradiation. Typically, the solutions were irradiated to a total dose of 1.0×10^5 Gy which converts approximately 5% of 5'-AMP to 8,5'-cycloAMP. Nitrous oxide, which was scrubbed of oxygen and acidic impurities by passage through 0.1 $mol \cdot dm^{-3}$ sodium dithionite and

0.1 mol·dm^{-3} sodium carbonate solutions, was used to double the yield of ·OH radicals (3) and to scavenge hydrated electrons which are known to promote phosphate release from 8,5'-cycloAMP (4). Products of irradiation and unchanged 5'-AMP were isolated by chromatography on a AG1-X8 (formate) anion exchange column (4.5 cm x 28 cm) using 0.4 N formic acid as the eluent flowing at 7.0 ml/min. For the quantitative radiolysis studies, 2.0 ml samples of 10^{-3} mol·dm^{-3} 5'-AMP in doubly distilled water were irradiated at various pH's. Perchloric acid or sodium hydroxide was used to adjust the pH. Following irradiation, the solutions were adjusted to 0.3 N HCl and heated at 100°C for 60 minutes during which time the large excess of 5'-AMP was hydrolyzed to leave three acid stable products which were quantified by means of high-pressure liquid chromatography (HPLC).

HPLC analyses were performed using a Milton Roy Minipump (46 - 460 ml/hr) with pulse damper (Bioanalytical Systems Inc.), a Rheodyne 7120 syringe loading sample injector, and a Tracor 970A variable wavelength detector set at 266 nm for nucleotide analysis and 259 nm for adenine release. A Spectra Physics 4100 computing integrator was used to record yields. For nucleotide analysis a Whatman Partisil 10/25 PXS SAX anion exchange column (4.6 mm x 25 cm) was used with an eluent of 0.015 mol·dm^{-3} K_2HPO_4 buffer (pH 3.1) flowing at 1.2 ml/min. For the measurement of radiation-induced adenine release, a Whatman Partisil 10/25 PXS SCX cation exchange column (4.6 mm x 25 cm) was used with an eluent of 0.05 mol·dm^{-3}

$NH_4H_2PO_4$ (pH 3.3) flowing at 0.75 ml/min. All HPLC analyses were performed at ambient temperature. For quantification of yields, the chromatographic systems were calibrated with authentic samples of 8,5'-cycloAMP, 8-hydroxyAMP and adenine.

Infrared spectra were recorded with a Unicam SP1000 IR spectrophotometer and ultraviolet spectra with a Beckman DU-7 UV/VIS spectrophotometer. Nuclear magnetic resonance (NMR) and mass spectrometry was carried out in the Spectroscopy Laboratory of the Chemistry Department, University of Alberta with a Brüker WH-200 200 MHz NMR spectrometer and a Kratos MS-50 mass spectrometer, respectively.

RESULTS

In the chromatogram of an irradiated solution of 10^{-3} mol·dm^{-3} 5'-AMP under the HPLC conditions used here, the peak for unchanged 5'-AMP completely masks the presence of one of the radiolysis products (peak I, Figure 1). This was revealed when the irradiated samples were hydrolyzed in 0.3 N hydrochloric acid as described previously (1). Under these conditions, the hydrolysis of unchanged 5'-AMP (with release of adenine) reveals the presence of three acid stable, UV-absorbing products (I, II and III, Figure 1). In addition to the three acid stable products, which are unchanged even after prolonged acid hydrolysis, there is an acid labile product which cochromatographs with the acid stable product III. The structure of the acid labile product has not been determined. Upon

identification of compounds I, II and III, their quantification was achieved with a good degree of precision by peak area integration of chromatograms as in Figure 1. The yield of 8,5'-cycloAMP as a function of pH is in good agreement with previous values (1).

The acid stable product (I) was chromatographically and spectroscopically identical to the (S)-epimer of 8,5'-cycloAMP studied previously (1,4-6). The NMR spectrum (Figure 2, Tables 1 and 2) of compound II is clearly that of the (R)-epimer of 8,5'-cycloAMP. In particular, there is no signal from the proton in the C(8) position of adenine which is lost in the cyclization process. In addition, the C(5') proton experiences a very small coupling to the C(4') proton (Table 2) which, because of stereochemical considerations, is just what is expected of the (R)-epimer (5-7). The doublet signal for the 5' proton is due to coupling with the phosphorus atom of the 5'-phosphate group and not to the C(4') proton, which appears as a broadened singlet at 4.92 ppm. The assignment of C(2') and C(3') protons (Table 1), which differs from an earlier assignment (8), was made on the basis of three observations. First, the lower field doublet of the two doublets corresponding to C(2') and C(3') protons is broadened relative to the higher field doublet in both (R)- and (S)-epimers of 8,5'-cycloAMP. This distinguishes the two protons in that one of them is experiencing a small additional coupling to an adjacent proton in the molecule. Secondly, the broadened doublet is at higher field in the (R)-epimer that in the (S)-epimer of 8,5'-cycloAMP (4.38 ppm vs. 4.71 ppm in D_2O and 4.21

ppm vs. 4.48 ppm in deuterated dimethyl sulfoxide), whereas the sharper doublet has a similar chemical shift in both epimers. As the only change in the (R)- and (S)-epimers is the orientation of the phosphate group which should affect the C(3') proton with the broadening being accounted for by a small coupling to the C(4') proton. Finally, in a spin decoupling experiment, it was found that saturation of the C(4') proton signal led to a sharpening of the broadened C(3') doublet by 27 per cent whereas the sharper C(2') doublet was further sharpened by only 6 per cent. These data reveal that the previous assignment (8) of C(2') and C(3') protons in 8,5'-cycloAMP, which is opposite to that recorded in Table 1, was made on the basis of insufficient evidence.

The third acid stable radiolysis product (product III, Figure 1) proved to be 8-hydroxyAMP upon comparison with an authentic sample prepared by the hydrolysis of 8-bromoAMP (2). In particular, the isolated radiolysis product cochromatographed with authentic 8-hydroxyAMP, possessed infrared signals at 1740 and 1710 cm^{-1} and a characteristic pH-dependent ultraviolet spectrum (λ_{max} 263.5 nm, 284 nm (shoulder) (pH 1)) and (λ_{max} 279.5 nm (pH 9)). Alkaline phosphate hydrolysis of compound III in pH 10.5 Sorensen's glycine buffer as described previously (5) gave a product which cochromatographed with 8-hydroxyadenosine formed by the alkaline phosphatase hydrolysis of authentic 8-hydroxyAMP.

We had shown previously (1) that the yield of 8,5'-cycloAMP is strongly pH-dependent. However, we have now found that not only

is the overall yield of 8,5'-cycloAMP pH-dependent, but that the relative yields of its (R)- and (S)-epimers are also pH-dependent (Figure 3). At pH 3, the (R)-epimer predominates while at pH greater than 7, the (S)-epimer predominates. It would appear that the HPLC system used in an earlier study (1) was incapable of resolving (R)- and (S)-8,5'-cycloAMP and that the combined yields of the epimers had been recorded. It is also clear that in the purification of 8,5'-cycloAMP in the earlier studies (1,5,6) the (S)-epimer had been obtained free of the (R)-epimer.

The pH-dependence of the yields of the two 8,5-cycloAMP epimers and that of 8-hydroxyAMP show an inverse relationship (Figure 4). At pH 5.0 where the yield of 8,5'-cycloAMP is at a minimum, that for 8-hydroxyAMP is close to a maximum. At pH 9.0 the situation is reversed and 8,5'-cycloAMP is at a maximum whereas the yield of 8-hydroxyAMP is low.

At low pH (less than 3) the yield of both 8-hydroxyAMP and 8,5'-cycloAMP decreases, but this is offset by an increased yield of adenine release (Figure 5). In fact, the sum of G values for the two 8,5'-cycloAMP epimers, 8-hydroxyAMP, phosphate release and adenine release over the pH range 2-9 is reasonably constant. Only at very high pH (> 10) does the combined yield of these products decrease (Figures 3-5 and Table 3).

The release of adenine from irradiated 5'-AMP (Figure 5) increases over the pH range (5 to 3) in which adenine is protonated (pK_2 = 3.9) and direct $^{\cdot}OH$ attack on adenine (8-hydroxyAMP formation)

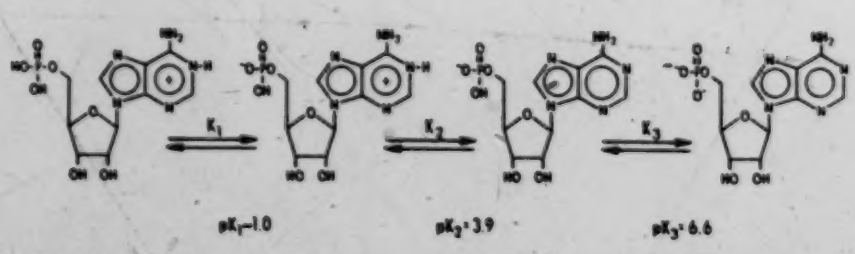
decreases. Adenine release is, however, very much less affected by changes in the ionization state of the phosphate group being relatively unchanged over the pH range 5 to 11 where the phosphomonoester is converted from the singly to doubly charged form ($pK_3 = 6.6$).

DISCUSSION

The distribution of ·OH attack within simple organic molecules is dependent on the presence or absence of unsaturation, the number and strengths of C-H bonds and, because of the electrophilic nature of ·OH, the presence or absence of charged groups such as ammonium or carboxylate ions in the molecule (9,10). All of these factors must be considered when ·OH attacks 5'-AMP or more complex systems such as nucleic acids. In the latter case, stereochemical factors could also be important. Pulse radiolysis studies have shown that the reaction rate of ·OH with 5'-AMP decreases at low pH (11). This is possibly due to the fact that protonation of adenine greatly decreases its rate of reaction with ·OH resulting in a smaller contribution from the adenine moiety to the overall reaction rate of 5'-AMP with ·OH at low pH. What is not clear from the pulse radiolysis studies of 5'-AMP is the effect that the changing rate of ·OH reaction at the base moiety has on the radiolysis of the sugar moiety. The steady-state radiolysis study reported here provides some insight to this question. The nucleotide analog, 8-hydroxyAMP can be considered a steady-state probe of ·OH reaction at the adenine

base in 5'-AMP in analogy with earlier studies with adenine (12-14) while 8,5'-cycloAMP formation (1,15), phosphate release (1) and adenine release can be considered probes of radiolysis events in the sugar moiety in a model compound which incorporates the basic features of structures occurring in nucleic acids. While 8,5'-cycloAMP is classified here as ribose damage because it originates with $^{\cdot}OH$ attack at the sugar moiety, it could also be classified as base damage and be of particular significance in double-stranded nucleic acids where the sugar phosphate backbone is exposed to and the bases shielded from direct $^{\cdot}OH$ attack (16).

It is noteworthy that the pH-dependence for the formation of 8,5'-cycloAMP parallels that for phosphate release from 5'-AMP [(1), Table 3]. This tends to rule out the possibility, on one hand, that an acid-base catalyzed hydrolysis of a radical intermediate forms the basis for the pH-dependence of phosphate release and, on the other hand, that pH has an effect on the cyclization process involving the C(5') radical and the C(8) position of adenine. The most logical explanation for the parallel pH dependency for these different end points is that hydrogen abstraction by $^{\cdot}OH$ radicals at the carbon bearing the phosphate group is the crucial step in both cases and that this process is dependent on the charged state of the phosphate group.



Phosphate group ionization cannot totally account for yields in the pH range of 3 to 5, however, because the monoionized phosphate group predominates at both pH 3 and pH 5 where a maximum and a minimum yield of phosphate release and 8,5'-cycloAMP formation occurs. In the earlier study (1) it was suggested that changing partial rate factors for ·OH attack not only at the carbon bearing the phosphate group, but also at the C(8) position of the adenine base could contribute to the pH-dependence observed for the radiolysis at the sugar moiety although direct evidence was lacking. The fact that the maximum in the yield of radiation damage at adenine (8-hydroxyAMP) coincides with a minimum in the yield of radiation damage at the ribose moiety (Figure 4 and 5 and Table 3, pH 5 data) now provides direct evidence for the basic correctness of the partial rate factor argument. As the adenine base deprotonates in the pH range of 2.9 to 4.9 (pK_2 3.9), ·OH attack at the C(8) position of adenine is increasingly favored over attack at the 5'-phosphate moiety with the result that there is an increase in damage at the adenine base. Following this line of reasoning, it might also appear that hydrogen abstraction by ·OH at a carbon bearing a phosphate group with a double negative charge is favored over attack at the C(8) position of the adenine base on the basis that the yield of 8-hydroxyAMP decreases while that of 8,5'-cycloAMP increases in the pH range of 5.6 to 7.6 over which the phosphate group becomes doubly charged (pK_3 6.6) while the adenine base remains in this uncharged state. However, the possibility that other products arising from ·OH attack

at C(8) of adenine such as the 5'-ribonucleotide derivatives of the acid-labile, ring-opened product, 4-amino-5-formamidopyrimidine, or of the hydrated adenine product, 6-amino-8-hydroxy-7,8-dihydroadenine, increase in yield coincidentally with the decrease in 8-hydroxyAMP yield over the pH range 5.6 to 7.6 cannot be ruled out (12-15).

Adenine release appears to be generally insensitive to the state of the phosphate group ionization and remains relatively constant over the pH range 5-11 (Figure 5). Adenine release is, however, very much more dependent on the ionization state of the adenine base. As noted above, protonation of the adenine base and its concomitant inactivation with respect to direct ·OH attack leads to an increase in ·OH attack at the ribose ring as measured by 8,5'-cycloAMP and phosphate release. An even more pronounced effect is seen in the shift to the type of ribose damage which leads to adenine release. The radiolysis mechanisms proposed for base release (17) indicate that this process originates primarily from ·OH attack at ribose carbons such as C(4') which do not carry an ionizable substituent under the conditions of the present experiment. A comparison of yields of 8,5'-cycloAMP, 8-hydroxyAMP and adenine release at acid, neutral and alkaline pH (Table 3) indicates that the shift in ·OH attack away from the protonated adenine base at low pH leads to a relatively greater increase in ·OH attack at positions on the ribose ring leading to adenine release than at the C(5') position which leads to 8,5'-cycloAMP formation. A possible

explanation for the apparent decrease of ribose damage and an increase in adenine release at low pH is that an acid-catalyzed hydrolysis of radical intermediates of the sort proposed for nucleotide degradation (17) favors adenine release when adenine is protonated. This possibility is under further investigation. It is clear that phosphate release from 5'-AMP parallels the formation of 8,5'-cycloAMP at low pH [(1) and Table 3] and so an increase in phosphate release cannot account for the relatively low yield of 8,5'-cycloAMP compared to the yield of adenine release over the pH range of 2-5.

In addition to the apparent pH-dependent changes in the site of $^{\cdot}OH$ attack predicated by the pK_a of moieties within 5'-AMP, there appear to be pH-dependent phenomena which only indirectly reflect acid-base equilibria in the nucleotide. For example, the shallow minimum in the 8,5'-cycloAMP yield around pH 8 does not correspond to pK_a's within 5'-AMP. It does correspond, however, to a slight increase in adenine release over this pH range (Figure 5) and it is tempting to speculate that these two phenomena are related. Finally, while the overall yield of 8,5'-cycloAMP is determined by acid-base equilibria in 5'-AMP, the change in the relative yields of the (R)- and (S)-epimers of 8,5'-cycloAMP as a function of pH indicates the presence of other factors. In the pH range where the phosphate group carries a single negative charge (pK_1 approximately 1.0) the (R)-epimer predominates. An examination of molecular models shows that the gauche-trans (18) configuration at C(5') which would lead to the (R)-epimer places the singly-charged 5'-phosphate group

in proximity to the oxygen atom contained in the ribose ring. It may be that intramolecular hydrogen bonding between the 5'-phosphate group and the ring oxygen at low pH favors a gauche-trans C(5') configuration and a predominance of the (R)-epimer of 8,5'-cycloAMP. Although an NMR study of pH-dependent conformation effects in nucleotides might have shed light on this possibility, a distinction between gauche-trans and trans-gauche configurations at C(5') was not possible (19).

In summary, the changes in $^{\cdot}OH$ attack in 5'-AMP are strongly pH-dependent in a manner which can be rationalized in terms of acid-base equilibria for moieties within 5'-AMP. On one hand, protonation of the adenine ring (pK_2 3.9) leads to a decrease in attack at the C(8) position of adenine and a concomitant increase in radiation damage to the ribose moiety as measured by the combined yields of phosphate release, 8,5'-cycloAMP formation and adenine release. On the other hand, a 5'-phosphate group bearing two negative charges activates the ribose ring with respect to both 8,5'-cycloAMP formation and phosphate release with a concomitant decrease in radiation damage at the C(8) position of the adenine base. Ionization of the phosphate group to the doubly negative state (pK_3 6.6) has little effect on the ribose damage which leads to adenine release. Although a doubly charged phosphate group will not occur in the phosphodiester structure of nucleic acids and the presence of other bases and supramolecular structures will have an effect, it is anticipated that the distribution of $^{\cdot}OH$ damage in irradiated nucleic

acids will be subject to the same general principles described here for the mononucleotide, 5'-AMP.

TABLE I

Proton Chemical Shifts

Nucleotide	Solvent	Shift (ppm) H(2)	H(8)	H(1')	H(2')	H(3')	H(4')	H(5')	H(5")	NH_2
5'AMP[a]	^2H_2O	8.01	8.38	6.06	4.76	4.50	4.40	4.10	4.10	—
(*S*)-8,5'-cyclo-AMP	^2H_2O	8.32	—	6.26	4.37	4.71	4.91	5.66	—	—
(*S*)-8,5'-cyclo-AMP	$(C^2H_3)_2SO$	8.16	—	6.02	4.04	4.48	4.83	5.60	—	7.42
(*R*)-8,5'-cyclo-AMP	^2H_2O	8.42	—	6.40	4.31	4.38	4.92	5.38	—	—
(*R*)-8,5'-cyclo-AMP	$(C^2H_3)_2SO$	8.22	—	6.11	3.97	4.21	4.91	5.30	—	7.60

[a] From Stolarski *et al.* (*8*).

TABLE II

Values of Proton Vicinal Coupling Constants[a]

Nucleotide	Solvent	Vicinal coupling constants (Hz) J(1',2')	J(2',3')	J(3',4')	J(4',5')	J(4',5")	J(5',5")	$J(^{31}P,5')$	$J(^{31}P,5")$
5'AMP[b]	^2H_2O	5.4	5.0	3.9	3.9	2.6	11.8	4.6	4.6
(*S*)-8,5'-cyclo-AMP	^2H_2O	0.0	—	0.0	5.9	—	—	9.4	—
(*S*)-8,5'-cyclo-AMP	$(C^2H_3)_2SO$	0.0	6.0	0.0	5.7	—	—	8.4	—
(*R*)-8,5'-cyclo-AMP	^2H_2O	0.0	5.8	0.0	0.3	—	—	—	9.0

[a] The designations 5' and 5" refer to the two configurations for the C(5') protons.
[b] From Sarma *et al.* (*19*).

TABLE III

Yield of Products from 5'-AMP Irradiated under Nitrous Oxide at Various pH's

Radiolysis product	G value pH 2	pH 5	pH 7	pH 10
(*S*)-8,5'-cyclo-AMP (I)	0.07	0.06	0.38	0.30
(*R*)-8,5'-cyclo-AMP (II)	0.13	0.08	0.14	0.10
8-Hydroxy-AMP (III)	0.15	0.54	0.14	0.01
Adenine release	0.89	0.44	0.50	0.47
Phosphate release	0.28[a]	0.20[a]	0.36[b]	0.36[a]

[a] Raleigh and Whitehouse, unpublished. Measured as described in Ref. (*20*).
[b] Ref. (*20*).

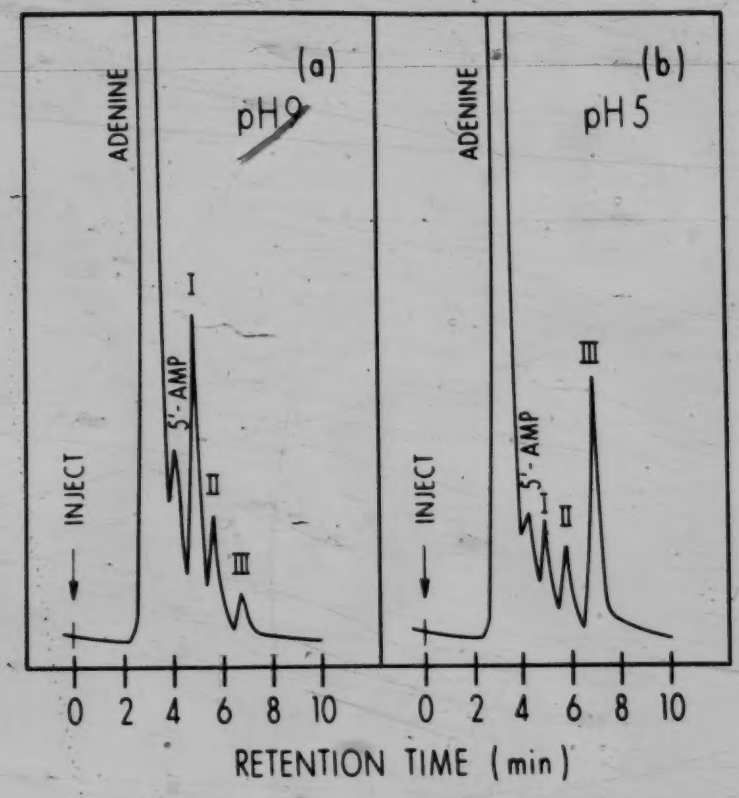


Figure 1: HPLC chromatograms of the acid-stable radiolysis products from 5'-AMP irradiated to 1000 Gy under N_2O at (a) pH 9 and (b) pH 5. Following irradiation, the irradiated solutions were adjusted to 0.3 N HCl and heated at 100°C for 60 minutes whereby 5'-AMP is hydrolyzed to adenine. Note for irradiation at pH 9 that (S)-8,5'-cycloAMP (peak I) and (R)-8,5'-cycloAMP (peak II) predominate while at pH 5 8-hydroxyAMP (peak III) predominates.

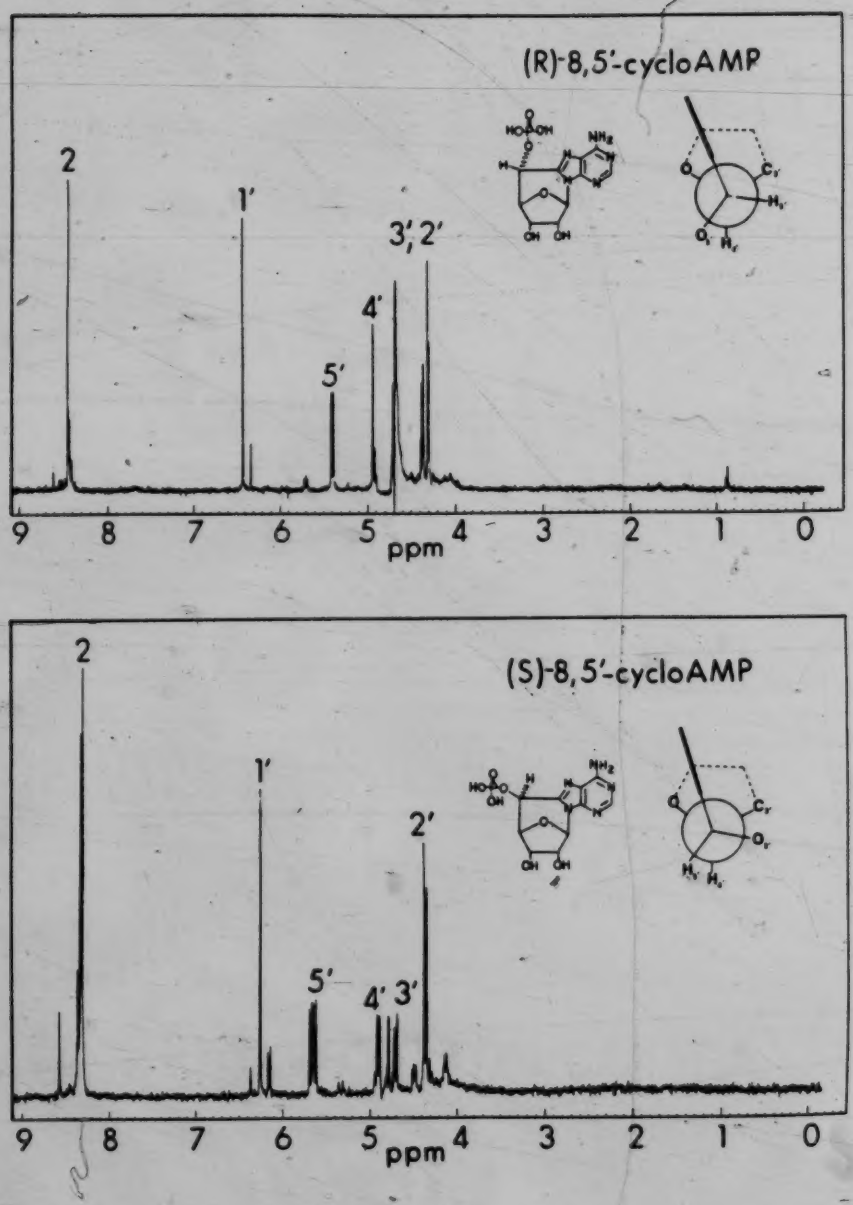


Figure 2: Nuclear magnetic resonance spectra (200 MHz) of (R)- and (S)- 8,5'-cycloAMP in D_2O. The peak at 4.8 ppm is due to partially repressed HDO formed from H_2O in the sample. Sample concentration was 10^{-2} mol·dm^{-3}. Chemical shifts and coupling constants are summarized in Tables 1 and 2, respectively.

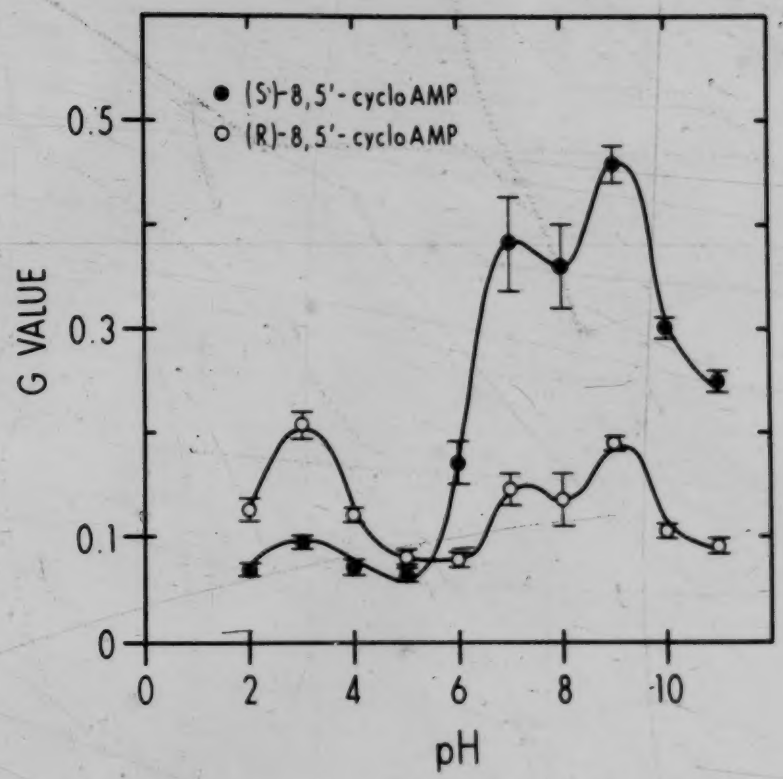


Figure 3: Yield of the (R)- and (S)-epimers of 8,5'-cycloAMP as a function of pH. Each point is the mean of three separate experiments (± standard error). The samples were irradiated to 1000 Gy under nitrous oxide.

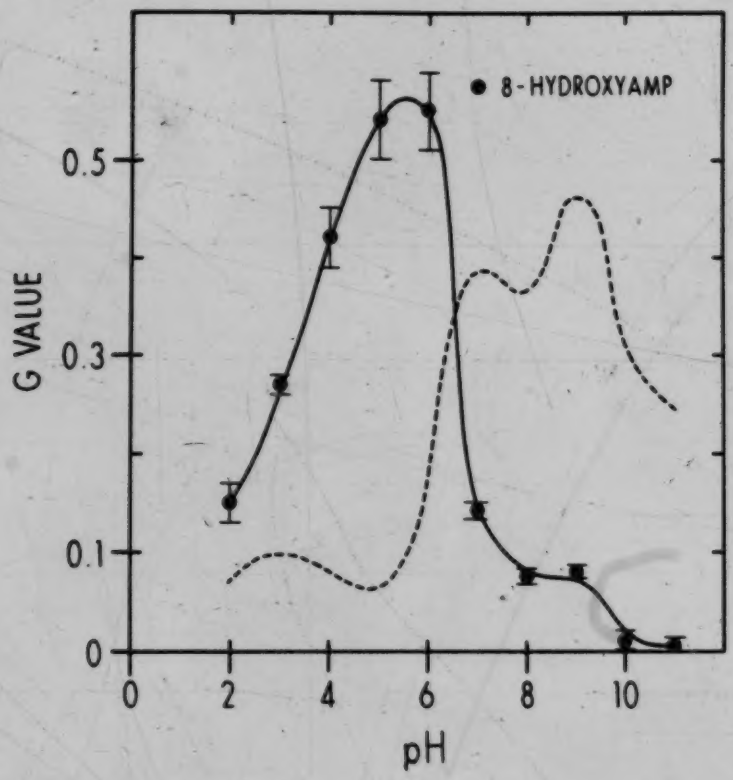


Figure 4: Yield of 8-hydroxyAMP as a function of pH. Radiolysis conditions were the same as in Figure 3. The dashed line is the pH profile for the yield of (S)-8,5'-cycloAMP from Figure 3.

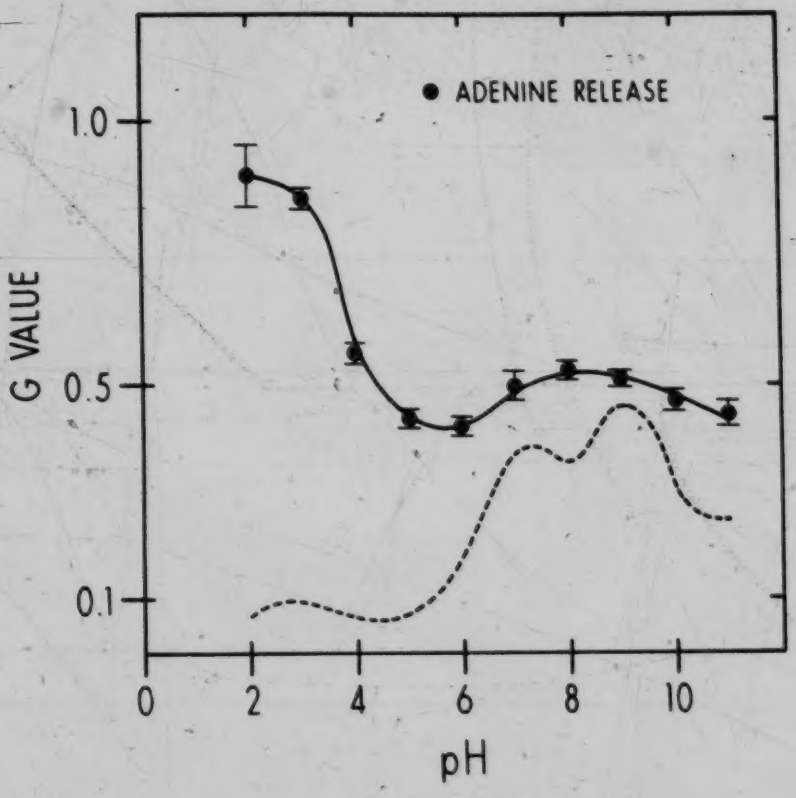


Figure 5: Yield of adenine release as a function of pH. Radiolysis conditions were the same as in Figure 3. The dashed line is the pH profile for the yield of (S)- 8,5'-cycloAMP from Figure 3.

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CHAPTER 3

RAPID SEPARATION OF ADENINE-CONTAINING RADIOLYSIS PRODUCTS USING ISOCRATIC, REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

INTRODUCTION

Due to the complexity of nucleic acids, analysis of a spectrum of specific radiation-induced chemical modifications is formidable. Our efforts have been directed to an understanding of the underlying radiation chemistry occurring within nucleic acids by using adenine-based compounds as models for radiolytic damage. In this context, we have demonstrated that upon irradiation of adenosine 5'-monophosphate (5'-AMP), under conditions favoring hydroxyl radical ('OH) attack, adenine release and the formation of altered nucleotides occurs. These altered nucleotides include three acid stable compounds: (R)- and (S)-8,5'-cycloadenosine 5'-monophosphate (8,5'-cyclcAMP) and 8-hydroxyadenosine 5'-monophosphate (8-hydroxyAMP) (1). In that earlier report, anion exchange high-performance liquid chromatography (HPLC) was used to measure the yield of the nucleotides following irradiation of 5'-AMP. A limitation of that HPLC system was that an acid hydrolysis step was necessary to destroy unchanged 5'-AMP prior to HPLC analysis because 5'-AMP overlapped significantly with (S)-8,5'-cyclcAMP during chromatography. Therefore, using that HPLC system we were unable to assay acid-labile products resulting

from the radiolysis of 5'-AMP.

In the present study, an isocratic, reversed-phase HPLC system was developed to separate adenine and adenine-containing monoribonucleosides. This system can be used to rapidly separate adenine, (R)- and (S)-8,5'-cycloadenosine (8,5'-cycloAdo) and 8-hydroxyadenosine (8-hydroxyAdo) from adenosine. This system can be extended to analyze the radiation-induced products from 5'-AMP and poly A without the need for acid hydrolysis of the irradiated solution. This is achieved by enzymatic hydrolysis in the case of 5'-AMP and chemical hydrolysis followed by enzymatic hydrolysis in the case of poly A, prior to direct injection of either hydrolysate onto the HPLC column. Not only are the previously mentioned compounds easily assayed with this system, but hitherto unidentified acid-labile products can now be studied.

METHODS AND MATERIALS

Chemicals

Polyadenylic acid (mol wt. 140-500 kdal), adenine, adenosine, 5'-AMP, 8-bromoAMP and alkaline phosphatase (Bovine Intestinal Mucosa, Type VII-S) were purchased from the Sigma Chemical Co. (St. Louis, MO) and used as received. All other chemicals were reagent grade and obtained from local suppliers. Authentic samples of (R)- and (S)-8,5'-cycloAdo were synthesized by a published procedure involving the photolytic cyclization of 2',3'-O-isopropylidene-5'-deoxy-5'-phenylthioadenosine (2). The

8-hydroxyadenosine product was prepared by the hydrolysis of 8-bromoAMP to 8-hydroxyAMP according to Ikehara et al. (3) followed by enzymatic hydrolysis to yield the nucleoside.

Irradiation

Samples of 5'-AMP, adenosine or poly A were irradiated as described previously (1,4). Briefly, samples were bubbled with nitrous oxide for 15 minutes prior to and then throughout the irradiation. Nitrous oxide was scrubbed free of oxygen by passage through a 0.1 $mol \cdot dm^{-3}$ sodium dithionite trap and scrubbed free of acidic impurities by passage through a 0.1 $mol \cdot dm^{-3}$ sodium carbonate trap. This procedure was used to double the yield of $^{\cdot}OH$ radicals (5) and to scavenge hydrated electrons which are known to promote phosphate release from 8,5'-cyclcAMP (6). Irradiation was performed in a Gamma-cell 220 ^{60}Co radiation source (Atomic Energy of Canada Ltd.) at a dose rate of 63.4 Gy/min as determined by Fricke dosimetry [$G(Fe^{3+}) = 15.6$ (7)].

Chemical and Enzymatic Hydrolysis

Following irradiation, poly A was chemically digested to mononucleotides using zinc-catalyzed hydrolysis (8). Digestion of poly A was followed by size exclusion chromatography using a 7.5 mm i.d. x 50 cm Varian MicroPak TSK G3000 SW column (Varian Assoc. Inc.). The eluent was 20 $mmol \cdot dm^{-3}$ K_2HPO_4 (pH 7.0) running at a flow rate of 1.4 ml/min and the products were monitored at 259 nm. The resultant mononucleotides were enzymatically hydrolyzed with alkaline phosphatase to the corresponding mononucleosides.

In the case of 5'-AMP radiolysis, the irradiated solutions were enzymatically hydrolyzed with alkaline phosphatase to the corresponding nucleosides.

Chromatographic Procedures

The analyses were performed on a Spectra-Physics SP8100 high-performance liquid chromatograph equipped with a SP8110 automatic sample injector coupled to a SP8440 variable wavelength detector and a SP4270 computing integrator. These components were interfaced with a LABNET module. The column used for the separation was a Waters μBondapak C_{18} (3.9 mm i.d. x 30 cm) which was protected by a guard column (2.1 mm i.d. x 10 cm) packed with Co:Pell ODS (Whatman Inc.). Isocratic elution was carried out using a methanol-ammonium formate eluent [HPLC grade methanol (Fisher Scientific Ltd.) and reagent grade ammonium formate (Mallinckrodt Inc.)] at a flow rate of 1.5 ml/min at ambient temperature. The chromatograms were monitored at 266 nm which is the maximum absorbing wavelength of the 8,5'-cycloAdo epimers. The pH of the eluent was adjusted with concentrated formic acid after the addition of methanol to the ammonium formate solution.

RESULTS

Analytical Conditions

The pH of the eluent has profound effects on the retention of adenine and the nucleosides studied (Figure 1). The capacity ratio $(k')^1$ changes less than 1.5 for (R)-8,5'-cycloAdo, and as

1. The capacity ratio (k') is defined as the difference in the retention time between the peak of interest and the solvent front divided by the retention time of the solvent front.

great as 3.3 for adenosine, as the pH of the eluent is raised from 3.0 to 5.5. The retention of adenosine is of particular concern in terms of separation, since this compound will be present at the highest concentration in experiments designed to quantitate products resulting from radiolysis. Therefore, a pH must be selected to ensure that good resolution is achieved between adenosine and either (S)-8,5'-cycloAdo or 8-hydroxyAdo. Lowering the pH of the eluent below 3.5 decreases the separation between (S)-8,5'-cycloAdo and adenosine whereas, increasing the pH of the eluent above pH 4.0 decreases the separation between 8-hydroxyAdo and adenosine. Therefore, an eluent with a pH in the range of 3.5 to 4.0 is necessary to separate these compounds. However, within this range the separation of (R)-8,5'-cycloAdo from adenine is greatest at pH 4.0. Therefore, pH 4.0 was selected as an optimum condition for the separation of the authentic samples and was used in subsequent studies.

The capacity ratio of adenine and adenine-based nucleosides was determined as a function of the methanol content of the mobile phase, keeping the overall ionic strength and the pH constant (Figure 2). As the concentration of methanol is raised from 5 to 15 percent, the capacity ratios of 8-hydroxyAdo, (S)-8,5'-cycloAdo and adenosine are markedly reduced, compared to the relatively small decrease in the capacity ratios of adenine and (R)-8,5'-cycloAdo, without significantly influencing the selectivity for these compounds. Therefore, adjusting the methanol content in the eluent is a convenient means of adjusting the total time required for each

chromatographic run. The concentration of ammonium formate in the eluent minimally affects the capacity ratios of 8-hydroxyAdo or (R)-8,5'-cyclcAdo over the concentration range 0.2 to 200 mmol·dm^{-3} (Figure 3). The capacity ratios of adenosine, (S)-8,5'-cyclcAdo and adenine increased less than 1.0 as the concentration of ammonium formate was decreased. Therefore, within the concentration range 0.2 to 200 mmol·dm^{-3}, the concentration of ammonium formate within the eluent was not found to be a useful parameter for adjusting the retention.

In the context of these studies, efficient separation of the authentic samples was achieved using an isocratic 10% methanol - 20 mmol·dm^{-3} ammonium formate (v/v) eluent at pH 4.0 as illustrated in Figure 4.

Analysis of Irradiated Mononucleotides and Polynucleotides

As a demonstration of the usefulness of the HPLC assay described in this report for radiation chemical studies, we have irradiated 5'-AMP and poly A to 1000 Gy, hydrolyzed the resultant products to monoribonucleosides, and injected the enzyme hydrolysates into the HPLC system. The pattern of nucleosides resulting from 5'-AMP radiolysis followed by enzyme digestion, reveals that (R)-8,5'-cycloAdo, adenine, (S)-8,5'-cycloAdo and 8-hydroxyAdo can be separated from unchanged adenosine and are thus amenable to quantitation (Figure 5a). A number of uncharacterized products (e.g. Products B and D) are also present and their identity is currently under investigation.

In the case of poly A, chemical and enzymatic hydrolysis is required to yield monoribonucleosides in a mixture which may be directly injected into the HPLC to yield the chromatographic pattern shown in Figure 5b. The radiolytic products (R)-8,5'-cycloAdo, (S)-8,5'-cycloAdo and adenine are separated from unchanged adenosine. The low yield of 8-hydroxyAdo from this mixture is related to differences in the radiation chemistry occurring in the polynucleotide as distinct from the mononucleotide and is not the result of selective chemical hydrolysis of this nucleoside as determined in control experiments with authentic 8-hydroxyAdo.

DISCUSSION

A high-performance liquid chromatographic procedure was developed to make possible the analysis of adenine and adenine-based ribonucleosides which are easily and rapidly separated in an isocratic, chromatographic system on a Waters μBondapak C_{18} column. This system has been used to separate the radiation-induced products of both 5'-AMP and poly A formed under conditions favouring hydroxyl radical attack. Irradiated solutions are hydrolyzed to the corresponding monoribonucleosides under mild conditions and the hydrolysates injected directly onto the HPLC column.

The pH, salt concentration and methanol content of the mobile phase were systematically varied to achieve maximal resolution of adenine, (R)- and (S)-8,5'-cycloAdo and 8-hydroxyAdo from adenosine. Consistent with previous results obtained by others studying the

chromatographic behavior of nucleosides and their heterocyclic bases (9-12), we have observed that : 1) the pH of the mobile phase influences the column capacity ratios and selectivity; 2) increasing the methanol content of the mobile phase reduces the column capacity ratios; and 3) the concentration of ammonium formate in the mobile phase has only a minimal effect on the retention of compounds. In the context of this work, we have also observed a remarkable separation of the (R)- and (S)-epimers of 8,5'-cyclcAdc which differ only in the spatial orientation of the hydroxyl group on the 5'-carbon of the ribose ring. In the (R)-epimer, the gauche-trans conformation about the C(4')-C(5') bond (13) places the C(5') hydroxyl group away from the ribose ring which appears to increase the hydrophilicity since the (R)-epimer has a much lower retention time than the (S)-epimer. Alternatively, a co-operative interaction of the C(5'), C(2') and C(3') hydroxyl groups with exposed silica sites on the reversed-phase column could account for greater retention times for the (S)-epimer and adenosine itself.

The radiation-induced decomposition of purine DNA model compounds has recently been reviewed by Cadet and Berger (14). A minimal amount of information is currently available to assess the relative importance of each type of lesion both in a qualitative and quantitative sense. Factors such as steric control imposed by the phosphodiester backbone in polynucleotides, the pH of the solution, and the oxygen status of the irradiated solution can profoundly influence the type and yield of products observed (1,4,15). The development of chromatographic systems such as the one described

here provides for the extension of our knowledge of effects of the microenvironment on product distribution in irradiated nucleic acids.

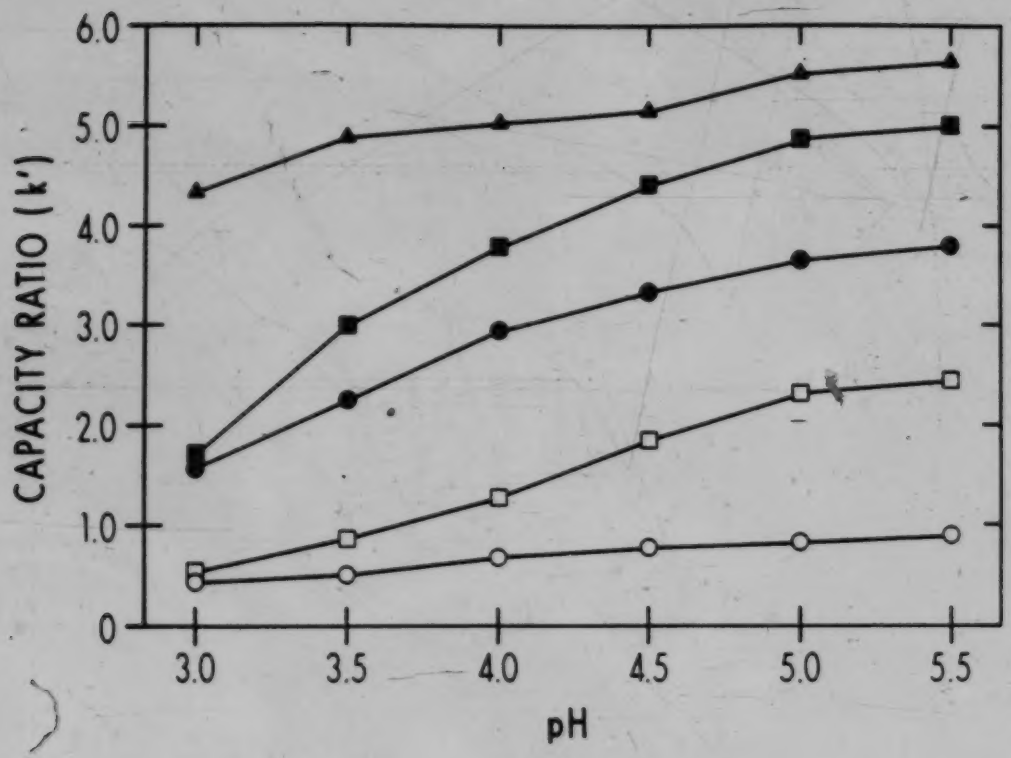

Figure 1: Dependence of the capacity ratio (k') on the pH of the mobile phase. Stationary Phase: µBondapak C_{18}; Mobile Phase: 10% MeOH - 20 mmol·dm^{-3} NH_4COOH. (8-hydroxyadenosine (▲); adenosine (■); (S)-8,5'-cycloadenosine (●); adenine (□); and (R)-8,5'-cycloadenosine (○)).

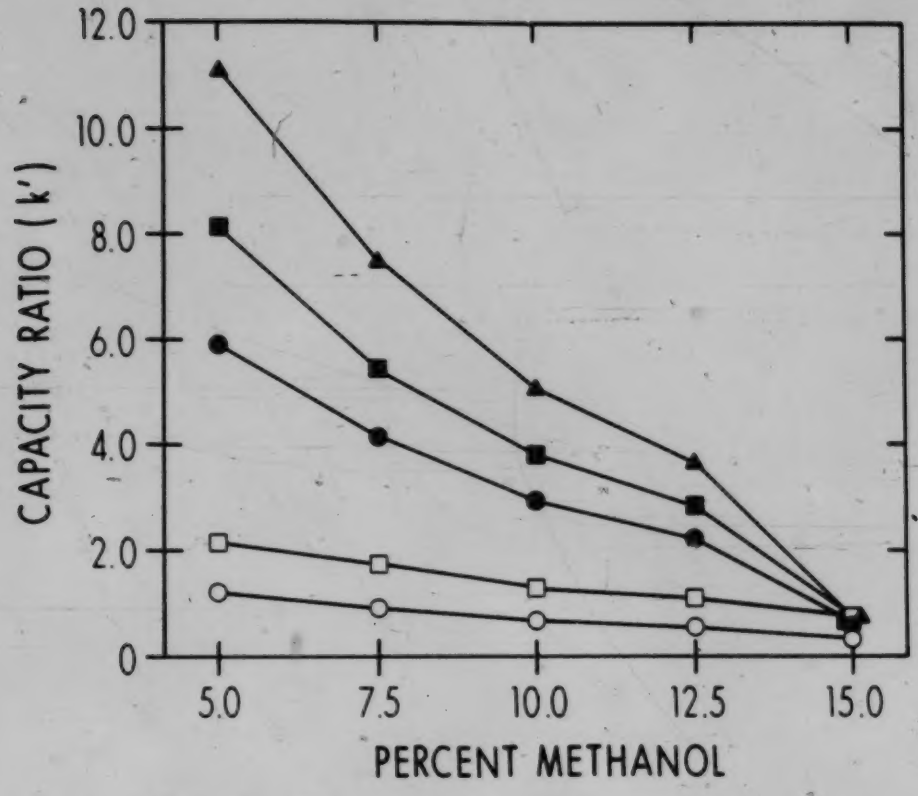

Figure 2: Effect of the methanol content in the mobile phase on the capacity ratio (k'). Stationary Phase: μBondapak C_{18}; Mobile phase: 5-15% MeOH - 20 $mmol \cdot dm^{-3}$ NH_4COOH (pH 4.00). (See Figure 1 for the identity of the compounds).

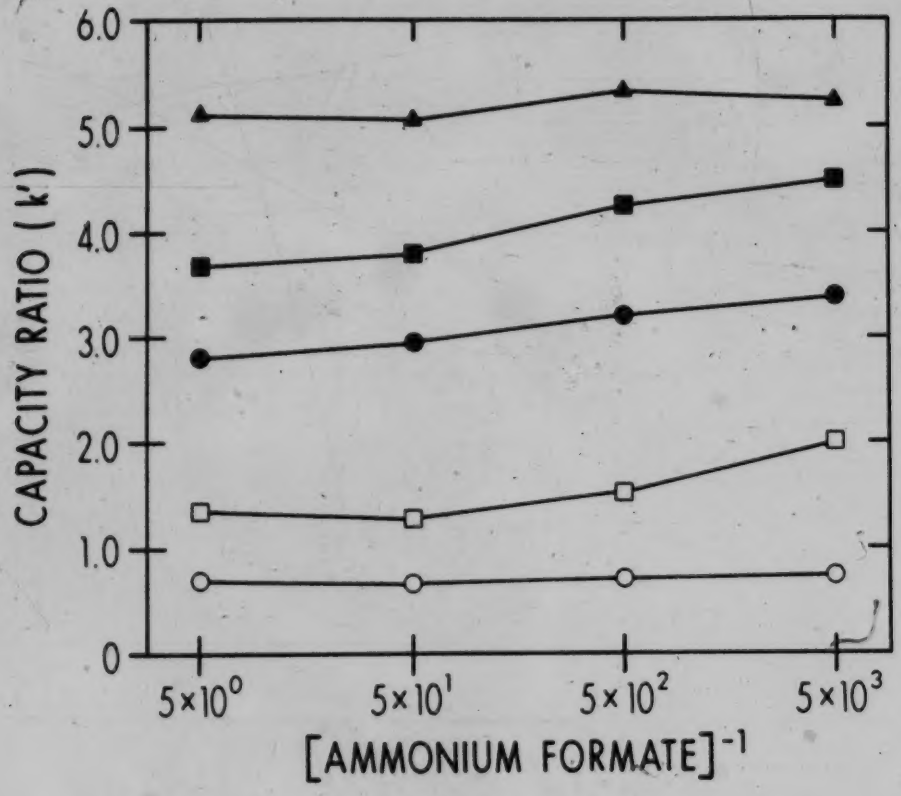

Figure 3: Dependence of the capacity ratio (k') on the concentration of ammonium formate in the mobile phase. Stationary Phase: μBondapak C_{18}; Mobile Phase: 10% MeOH - (0.2 - 200) mmol·dm^{-3} NH_4COOH, (pH 4.00). (See Figure 1 for the identity of the compounds).

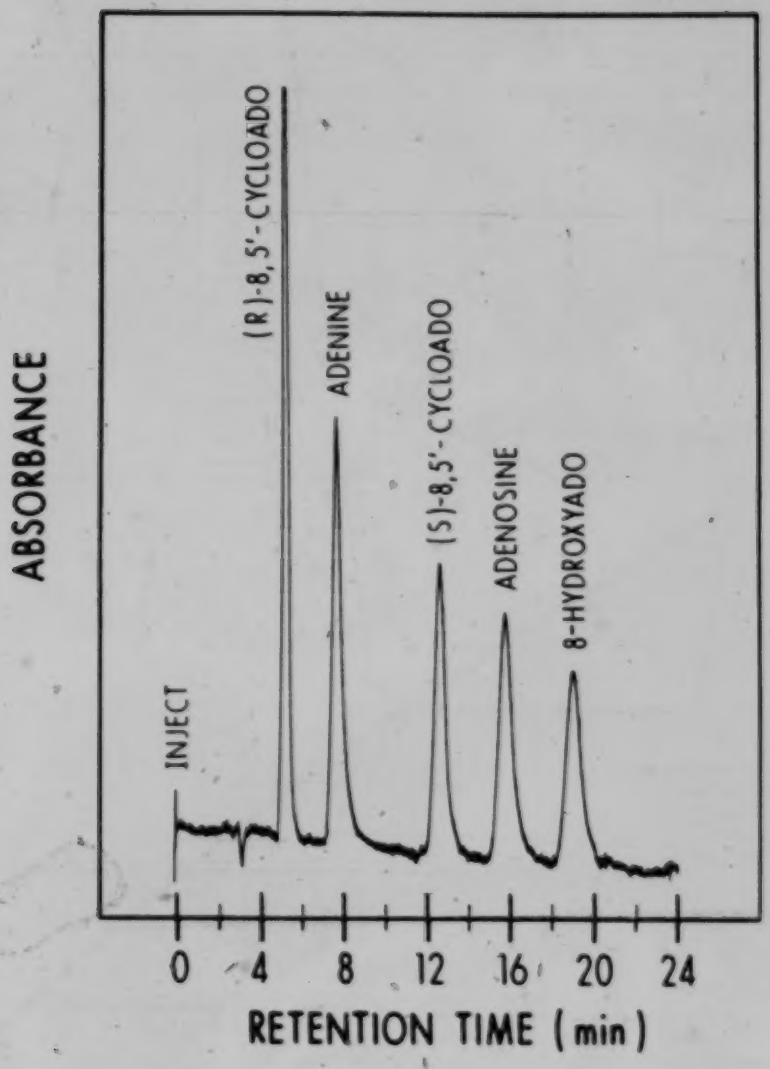

Figure 4: Elution pattern obtained for the separation of authentic samples. Stationary Phase: μBondapak C_{18}; Mobile Phase: 10% MeOH - 20 mmol·dm^{-3} NH_4COOH, (pH 4.00).

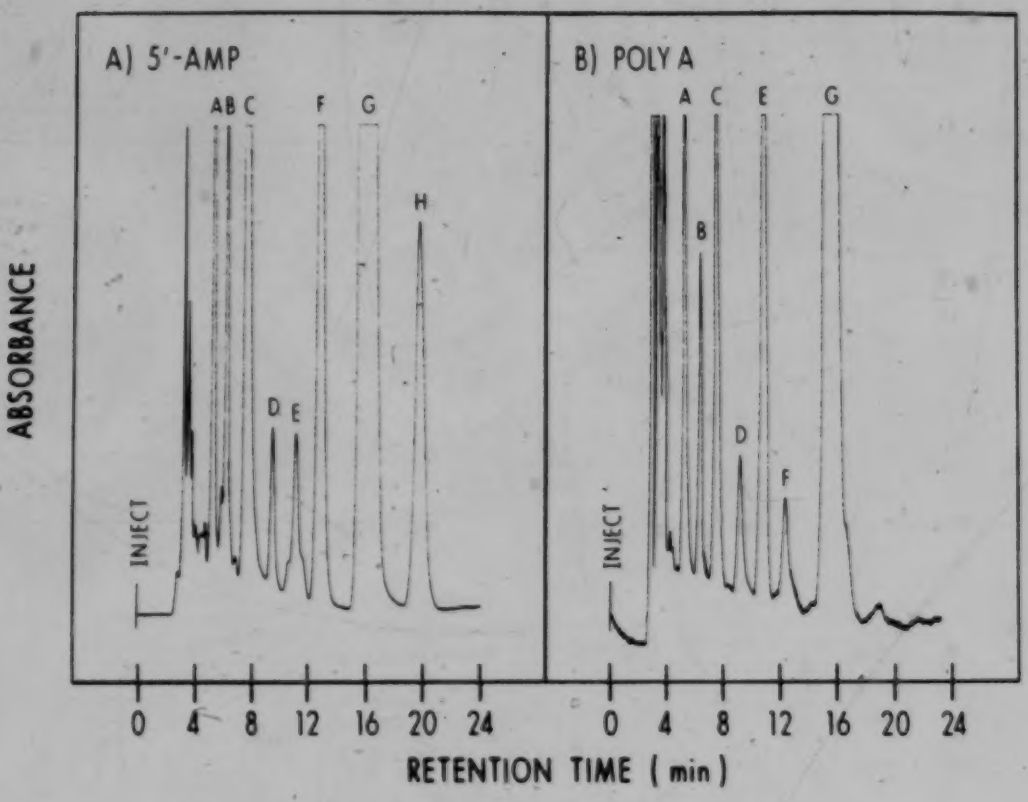

Figure 5: Elution pattern obtained from the chromatography of 5'-AMP and poly A solutions irradiated at 1 x 10^{-3} mol·dm^{-3} to 1000 Gy under N_2O (pH 7.00). Compounds: A) (R)-8,5'-cycloadenosine, C) adenine, F) (S)-8,5'-cycloadenosine, G) adenosine, H) 8-hydroxyadenosine. The identities of other components in the HPLC profile have not been determined. HPLC conditions described in Figure 4.

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CHAPTER 4

OXYGEN-DEPENDENCE OF PRODUCT FORMATION IN IRRADIATED ADENOSINE 5'-MONOPHOSPHATE

INTRODUCTION

The presence of molecular oxygen is an important factor governing the sensitivity of cells to ionizing radiation. The radiation sensitivity of mammalian cells increases with oxygen concentrations in the range of 0.3 to 30 $\mu mol \cdot dm^{-3}$ with a K_m (concentration required to give half-maximal response) of 3 $\mu mol \cdot dm^{-3}$ (1). This oxygen effect has been of considerable heuristic value in investigations of the molecular mechanisms of the biological effects of ionizing radiations. Molecular oxygen is known to alter the distribution of molecular damage in irradiated nucleic acids and their constituents (2-7) but few studies of the oxygen-dependence of molecular events over the 0.3 to 30 $\mu mol \cdot dm^{-3}$ oxygen concentration range have been performed which might tie the radiobiological effect to more specific molecular changes. As an initial model study, the oxygen-dependency of product formation has been assessed for a simple mononucleotide, adenosine 5'-monophosphate (5'-AMP), with emphasis on three classes of molecular products. 8,5'-Cyclonucleotides (8-16) represent one class of damage

1. A version of this chapter will be submitted for publication. Fuciarelli, A.F., Koch, C.J. and Raleigh. J.A. Oxygen-dependence of product formation in irradiated adenosine 5'-monophosphate. Radiat. Res. (1987).

characterized by maximal yield in the absence of oxygen and complete inhibition at high oxygen concentration. The second class of damage, 8-hydroxynucleotide formation (11,15,18), is similar to the first class of damage in that product formation decreases with increasing oxygen concentration, but differs in that complete inhibition of product formation does not occur at high oxygen concentrations. The third class of damage, exemplified by radiolytic adenine release (3), is one in which product yield is much less dependent on the oxygen status during irradiation. In addition to the characterization of different classes of damage on the basis of their oxygen-dependency, the yield of the 8-hydroxynucleotide and a component of adenine release were found to increase when the irradiation was performed in the presence of increasing concentrations of hydrogen peroxide.

MATERIALS AND METHODS

Reagents

Authentic samples of (R)- and (S)-8,5'-cycloadenosine were synthesized by a published procedure involving the photolytic cyclization of the 2',3'-O-isopropylidene derivative of 5'-deoxy-5'-phenylthioadenosine (19). The final products of this procedure were identified by combined gas chromatography - mass spectrometry analysis (Dr. M. Dizdaroglu, personal communication) and were found to be identical to (R)- and (S)-8,5'-cycloadenosine 5'-monophosphate (8,5'-cycloAMP) hydrolyzed to the corresponding

nucleoside obtained from the radiolysis of adenosine 5'-monophosphate (5'-AMP) (11). Authentic 8-hydroxyadenosine was synthesized according to a published procedure (20) and was characterized by infrared and ultraviolet spectroscopy and mass spectrometry. Alkaline phosphatase (Bovine Intestinal Mucosa, Type VII-S), 5'-AMP, hydrogen peroxide, dimethyl sulfoxide (DMSO) and catalase (Bovine Liver) were obtained from the Sigma Chemical Co. (St. Louis, MO) and used without further purification. Other chemicals were chromatographic or reagent grade and were obtained from local suppliers. The gas mixtures were prepared using pure nitrogen (< 10 p.p.m. O_2) and oxygen gases (Medigas, Edmonton, AB).

Preparation of Gas Mixtures

Gases containing various gas-phase oxygen concentrations were mixed prior to each irradiation. Typically a gas cylinder was evacuated and partially filled with air. Nitrogen gas was then pumped in to achieve the desired oxygen concentration. The mixed gas was allowed to pass through a flow regulator (Matheson, Model 621 PBV 0-15) to attain a reproducible flow rate of 588 ml/min which was proportioned to six solutions of 5'-AMP. These solutions were bubbled prior to irradiation until equilibrium was achieved (at which time the irradiation was initiated) and then throughout the irradiation interval.

Oxygen concentrations were measured by means of a modified Clarke electrode and specially constructed electronics (1). Both

the gas phase and liquid phase oxygen concentration were measured; the latter measurement being performed in a specially designed porcelain holder in which the position of the oxygen sensor was fixed. The liquid-phase oxygen concentration was measured in a solution of 5'-AMP prior to irradiation in order to ensure that equilibrium was achieved, then during radiolysis in order to establish the new equilibrium value attained in the presence of radiochemical oxygen consumption, and, finally, following irradiation in order to ensure that the liquid-phase oxygen concentration recovered in the expected way.

Irradiation

Irradiations were performed in a Gammacell-220 ^{60}Co gamma radiation source (Atomic Energy of Canada Ltd.) at a dose rate of 25 Gy/min as measured by Fricke dosimetry [$G(Fe^{3+}) = 15.6$, (21)]. Solutions of 5'-AMP (1×10^{-3} mol·dm^{-3}, pH 7.0) were made up in distilled water which had been purified and deionized before use by passage through a Barnstead three module NANOpure water purification system. The temperature of the solutions during radiolysis under the conditions described was 23°C.

High-Performance Liquid Chromatography

High-performance liquid chromatography was performed on a Spectra Physics system incorporating a SP 8100 liquid chromatograph, SP 8110 automatic sample injector, SP 8440 variable wavelength detector

set at 266 nm, and a SP 4270 computing integrator. A reversed-phase Waters μBondapak C_{18} column (3.9 mm x 300 mm) was used for the chromatography with an eluent of 7.5 per cent methanol in 20 $mmol \cdot dm^{-3}$ ammonium formate (pH 4.0) at a flow rate of 1.0 ml/min. Irradiated solutions of 5'-AMP were hydrolyzed with alkaline phosphatase as described previously (15). Adenine, adenosine, (R)- and (S)-8,5'-cycloadenosine and 8-hydroxyadenosine were identified as radiolysis products by cochromatography with authentic samples and the yields of these products were quantified with reference to calibration curves using authentic samples.

Establishing the Role of Hydrogen Peroxide

Hydrogen peroxide-related phenomena were investigated by addition of hydrogen peroxide to nitrogen-saturated aqueous solutions of 5'-AMP or by depletion of hydrogen peroxide by catalase from oxygen-saturated aqueous solutions of 5'-AMP. In the first case, solutions containing 5'-AMP (1×10^{-3} $mmol \cdot dm^{-3}$) in the presence of various concentrations of hydrogen peroxide (3.0 to 750 $\mu mol \cdot dm^{-3}$) were adjusted to pH 7.0, saturated with nitrogen (as described previously) and irradiated to 1000 Gy at a dose rate of 60.7 Gy/min. A second series of solutions of 5'-AMP (1×10^{-3} $mmol \cdot dm^{-3}$) was prepared containing in parallel sets, native and denatured catalase (26 ng/ml to 325 μg/ml). Catalase was denatured by lowering the pH into the acidic range and then returning the pH to neutrality. Solutions containing denatured catalase showed no evidence for the

liberation of oxygen following addition of high concentrations of hydrogen peroxide. The aqueous solutions of 5'-AMP (pH 7.0) were saturated with oxygen (as described previously) and irradiated to 1000 Gy at a dose rate of 60.7 Gy/min. Following irradiation, samples were treated with alkaline phosphatase and the hydrolysates were analyzed by reversed-phase HPLC as previously described.

RESULTS

One source of oxygen depletion in radiation chemical studies is radiochemical oxygen consumption - a process dependent on the dose rate. At reduced dose rate, radiolytic oxygen consumption is offset by oxygen diffusion and an equilibrium between gas and liquid phases can be attained (22,23). The characteristics of this equilibrium can be assessed by directly monitoring the partial pressure of oxygen in the liquid phase during radiolysis. In this manner, the oxygen concentration in the liquid phase at equilibrium at a dose rate of 25 Gy/min can be assessed with reference to the oxygen concentration of the gas phase when the gas is bubbled continuously into the solution containing the substrate (i.e. 5'-AMP, Figure 1). As indicated previously (1), at high gas-phase oxygen concentrations in a system that consumes oxygen, the difference in oxygen concentration between gas and liquid phases is small at high concentrations but continues to increase as the gas-phase oxygen concentration decreases (Figure 1). Radiochemical oxygen consumption resulting from the high dose rate used in these experiments, coupled

with the inability to introduce the gas into the solution because of excessive turbulence, prevented studies at equilibrium liquid-phase oxygen concentrations below 0.15%.

The formation of (R)- and (S)-8,5'-cycloAMP in irradiated solutions of 5'-AMP is a process that occurs in the absence of oxygen (8,9,11). The yield of (R)- and (S)-8,5'-cycloAMP under 0% (100% N_2), 0.15% and 0.30% oxygen concentrations was linear over the dose range studied (Figure 2). Consistent with an earlier report (11), the yield of the (S)-epimer predominated over that of the (R)-epimer at neutral pH. The formation of 8,5'-cycloAMP decreased as a function of increasing oxygen concentration (Figures 2 and 3) and was completely inhibited at a liquid-phase oxygen concentration of 1.62% with an estimated K_m of 0.08% (Figure 3).

In the case of 8-hydroxyAMP formation in irradiated solutions of 5'-AMP, the yield increased as a function of decreasing oxygen concentration (Figure 4). With an oxygen concentration of 100% the G value for product formation was 0.09 which increased to G = 0.16 in a solution saturated with 0.3% pO_2. However, under 100% nitrogen, 8-hydroxyAMP formation was undetectable in the dose range studied (Figure 4).

Radiolytic adenine release from 5'-AMP is apparently less susceptible to changes in oxygen concentration (Figure 4). The G value for adenine release varies from 0.28 (100% N_2) to 0.46 (100% O_2). Data from oxygen concentrations intermediate to these limits vary systematically in this range but were omitted from the figure for clarity.

The absence of 8-hydroxyAMP in irradiated nitrogen-saturated aqueous solutions of 5'-AMP prompted further study into the nature of this reaction. The involvement of hydroxyl radicals in the formation of this product is suggested by the observation that DMSO inhibits product formation in a concentration-dependent manner which is similar to inhibition of 8,5'-cyclonucleotide formation (Fuciarelli, unpublished). The role of hydrogen peroxide in promoting 8-hydroxyAMP formation was then investigated since hydrogen peroxide formation cannot be measured in nitrogen-saturated solutions yet the molecular yield of hydrogen peroxide is relatively high in oxygen-saturated solutions [G (H_2O_2) = 3.0; (24)]. To test the hypothesis that hydrogen peroxide might contribute in some way to product formation, a series of 5'-AMP solutions was prepared with increasing concentrations of hydrogen peroxide saturated with nitrogen and irradiated to 1000 Gy. With respect to 8-hydroxyAMP formation, the yield was found to increase as a function of hydrogen peroxide concentration (Figure 5). In the course of this study, a component associated with radiolytic adenine release (revealed by subtracting the yield of adenine release in solutions that did not contain additional hydrogen peroxide from the yield of adenine release in solutions which contained additional hydrogen peroxide) was observed to increase as a function of hydrogen peroxide concentration over the same range and with a G value similar to that for 8-hydroxyAMP formation (Figure 5). In contrast to these observations, changes in the yields of the (R)- and (S)-epimers of 8,5'-cycloAMP were

not apparent with increasing concentrations of hydrogen peroxide ($G_{(R)} = 0.08$ and $G_{(S)} = 0.31$).

DISCUSSION

The ability to alter the radiosensitivity of cells as a function of the availability of molecular oxygen during irradiation is a well documented phenomenon. Oxygen may affect either the repair processes (25) or the type and yield of specific molecular damage occurring in irradiated nucleic acids (2-7). Studies of the effect of oxygen on product formation in irradiated nucleic acids, both qualitatively and quantitatively, may serve to identify the types of damage that repair processes have to deal with in order to avoid mutation, carcinogenesis, aging and cell lethality. As a first step towards assessing the relevance of molecular lesions with reference to the radiobiological oxygen effect, the oxygen dependence of product formation in the purine mononucleotide adenosine 5'-monophosphate has been measured.

The oxygen-dependence of 8,5'-cyclonucleotide formation represents one class of damage characterized by maximal yield in the absence of oxygen and complete inhibition of product yield with increasing liquid-phase oxygen concentrations. The mechanism leading to 8,5'-cyclonucleotide formation from purine mononucleotides is better understood than those leading to most types of radiation-induced molecular modifications. The process is initiated by hydroxyl radical attack at the C(5') carbon of the ribose ring

followed, in the absence of oxygen, by addition of the C(5') radical to the C(8) position of the purine base and a one electron oxidation to form the 8,5'-cyclonucleotide moiety (8,9,11,12,14,15). The involvement of the hydroxyl radical in product formation is implied by the observation that product yield is increased in nitrous oxide as compared to nitrogen-saturated solution and that hydroxyl radical scavengers inhibit product formation (9,12). Inhibition of product formation, presumably by scavenging the C(5') radical intermediate, occurs with increasing liquid-phase oxygen concentrations with a K_m of 0.08% pO_2 and complete inhibition at 1.62% pO_2 (Figures 2 and 3). The oxygen effect for mammalian cell radiosensitivity is of the same order with a K_m of 0.3% pO_2 and complete radiosensitivity is achieved with liquid-phase concentrations exceeding 3% pO_2. If the radiobiological K_m were to be associated with specific molecular events, then the type of chemistry at the sugar phosphate backbone leading to the inhibition of cyclonucleotide formation would seem to qualify.

The second class of damage considered is 8-hydroxynucleotide formation for which the oxygen dependence is much more complex than that seen for 8,5'-cyclonucleotide formation. Oxygen is required for 8-hydroxynucleotide formation but its diminished yield at high O_2 concentrations is puzzling (Figure 4). A unique feature of 8-hydroxynucleotide formation is that there is complete inhibition in nitrogen-saturated solution, yet this product is observed in nitrous oxide-saturated (11,15) and nitrogen-saturated solutions

containing at least 0.5 mmol·dm^{-3} hydrogen peroxide (Figure 5). However, hydrogen peroxide itself does not produce 8-hydroxyAMP, since this product was not detected in unirradiated solutions of 5'-AMP containing similar concentrations of hydrogen peroxide. Furthermore, the addition of catalase (an enzyme known for its ability to destroy hydrogen peroxide), does not reduce product yield in solutions saturated with oxygen (results not shown). However, incubation of purines with hydrogen peroxide for long times (i.e. 5 days) has been known to yield similar products as observed in radiolysis (26, W.F. Blakely, personal communication).

The third class of damage, as represented by radiolytic adenine base release, is relatively independent of oxygen status in that the ratio of G values for radiolysis in oxygen- as compared to nitrogen-saturated solution is 1.64 (Figure 4). Adenine release from purine nucleotides(sides) is a relatively complex process and the mechanisms leading to base release are numerous and varied (2,3). In addition, hydrogen peroxide was observed to increase radiolytic adenine release in irradiated solutions of 5'-AMP with yields on the same order as observed with 8-hydroxynucleotide formation (Figure 5).

Qualitative and quantitative variations in product yield as a function of the presence or absence of molecular oxygen have been previously reported for phosphate release or phosphodiester cleavage in nucleic acids. In the case of irradiated, nitrogen-saturated solutions of DNA, Bopp and Hagen (27) reported a predominance of

3'-cleavage resulting in 5'-phosphate termini, whereas under oxygen the extent of 3'-cleavage was significantly reduced (27-29). Similarly, 3'-nucleotide bond cleavage was found to diminish in the presence of oxygen relative to nitrogen-saturated solutions in the case of adenine mononucleotides (30) and dinucleotides (31). On the other hand, 5'-nucleotide bond cleavage was observed to increase for mononucleotides (30), yet remain relatively constant for dinucleotides (31). The range of oxygen concentration over which the changes observed in phosphate release or phosphodiester cleavage is unknown.

The literature is fragmentary and incomplete concerning the yield of specific molecular modifications of adenine-containing nucleic acid constituents other than those reported here. For example, the 4-amino-5-formamido-6-(ribosyl)aminopyrimidine (FAPy) has been reported in nitrogen-saturated solutions of adenine (32,33), 2'-deoxyadenosine (34), polydeoxyadenylic acid (35) and DNA (34). In the case of oxygen-saturated irradiated solutions, Chetsanga and Grigorian (34) report inhibition of FAPy formation in 2'-deoxyadenosine, but formation in DNA; the latter finding also reported by Bonicel et al. (36) However, problems with quantitation of product yield in the report of Chetsanga and Grigorian (34) as discussed by Cadet and Berger (6), make these data unreliable and therefore the product yield in the presence or absence of oxygen cannot be assessed with confidence. Preliminary data from high-performance liquid chromatography - mass spectrometry studies

of adenine-based compounds in our laboratory suggest that the FAPy derivative is formed in nitrogen- and oxygen-saturated solutions of adenine, 3' and 5' adenine-based nucleotides and nucleosides of the ribose and deoxyribose series (Fuciarelli, unpublished) and in polyadenylic acid (16).

Changes in several types of radiation-induced modifications to DNA have previously been shown to occur within a very narrow range of oxygen concentrations just slightly lower than that range observed for cell survival. In mammalian systems, DNA single-strand break formation is increased 2.5 - 4 fold when the irradiation is performed under oxygen as compared to nitrogen (37-45). The K_m for DNA single-strand break induction was observed to be less than 20-fold lower than the K_m observed with cell survival for mammalian (42,46) and bacterial systems (47). This observation may be due to a difference between the extracellular oxygen concentration and the effective concentration of oxygen surrounding DNA. However, no data has been found to substantiate or refute this possibility. In the case of radiation-induced DNA-protein crosslinks, an increase in the yield of DNA-protein crosslinking was observed in hypoxic, as compared to oxygenated, environments for mouse L and V79 cells (45), human fibroblasts (48), V79 fibroblasts (49) and CHO cells (46). For DNA-protein crosslink formation the K_m was also reported to be slightly lower (less than a factor of 5) than the K_m for cell survival in the CHO system (46). In addition to these data, the K_m of 8,5'-cyclonucleotide formation is lower (K_m = 0.08% pO_2) (Figure

2) than the K_m for cell survival [K_m = 0.3% pO_2 (1)]. The significance of this pattern remains open for question.

In summary, the oxygen-dependence of product formation was assessed in irradiated 5'-AMP as a function of the liquid-phase oxygen concentration. Three classes of damage were identified on the basis of their oxygen dependency as represented by 8,5'-cyclonucleotide formation, 8-hydroxynucleotide formation and radiolytic adenine release, respectively. Major changes in product yield occurred in the same oxygen concentration range as did major changes observed by others in cell survival, single-strand breaks and DNA-protein crosslinks. In addition, hydrogen peroxide present at the time of irradiation increased the yield of 8-hydroxynucleotide formation and a component of radiolytic adenine release to the same extent whereas the yield of 8,5'-cyclonucleotide formation remained constant.

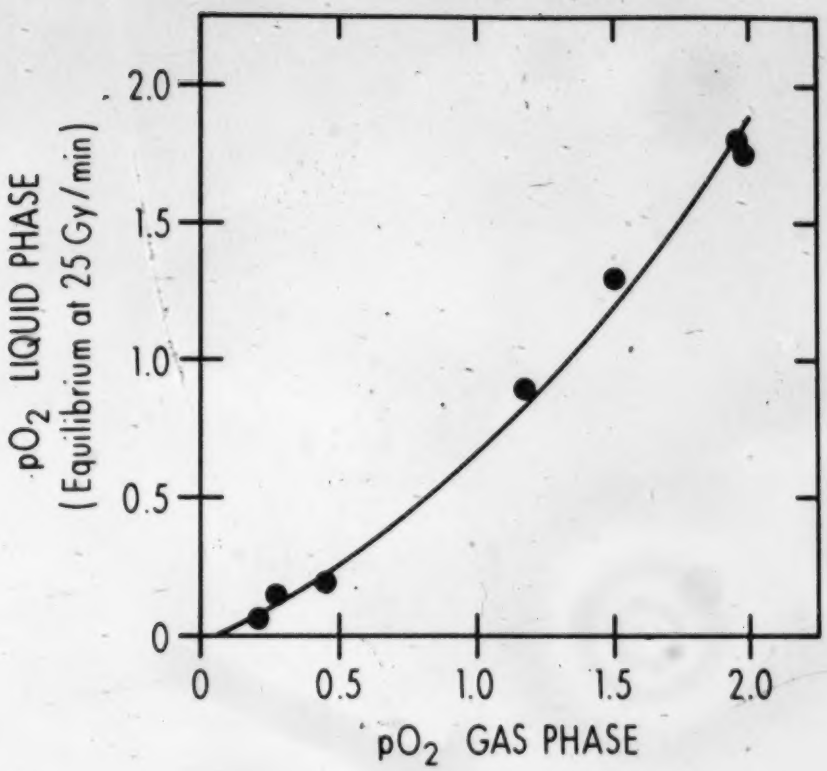

Figure 1: Oxygen concentration obtained in aqueous solutions of 5'-AMP at equilibrium at a dose rate of 25 Gy/min as a function of the oxygen concentration in the gas phase.

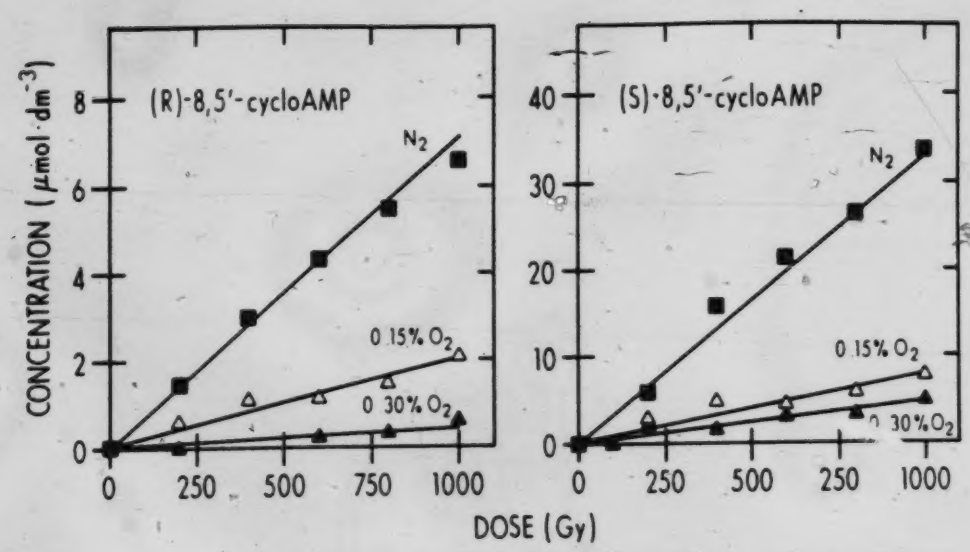

Figure 2: Yield of (R)- and (S)-8,5'-cycloAMP as a function of dose for various liquid-phase oxygen concentrations.

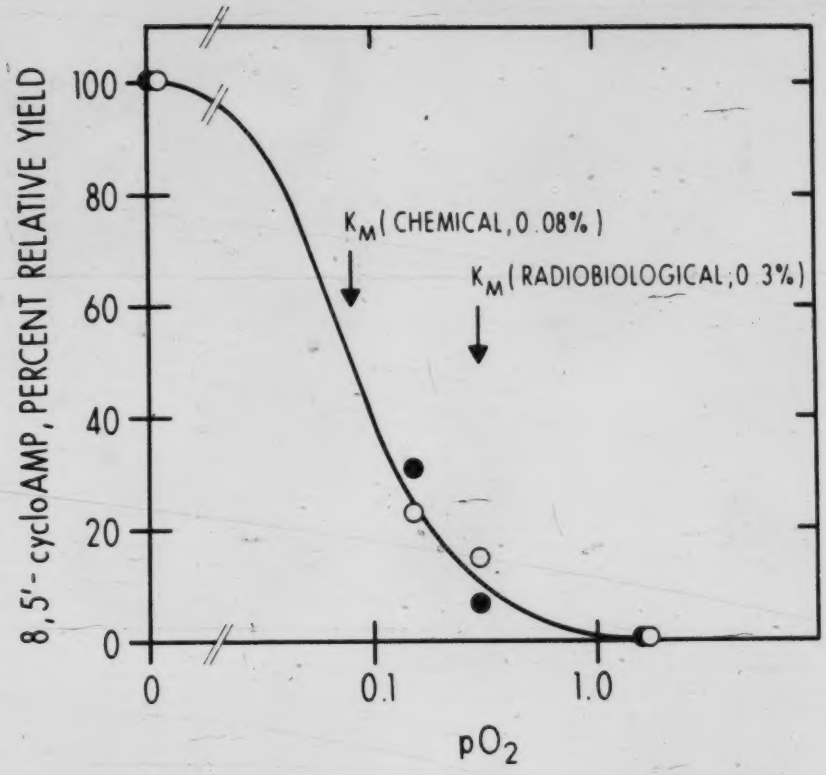

Figure 3: K_m for (R)- (●) and (S)- (○) 8,5'-cycloAMP formation. Data represents the relative yields of product at 1000 Gy plotted as a function of the liquid-phase oxygen concentration taken from regression analysis of data plotted in Figure 2.

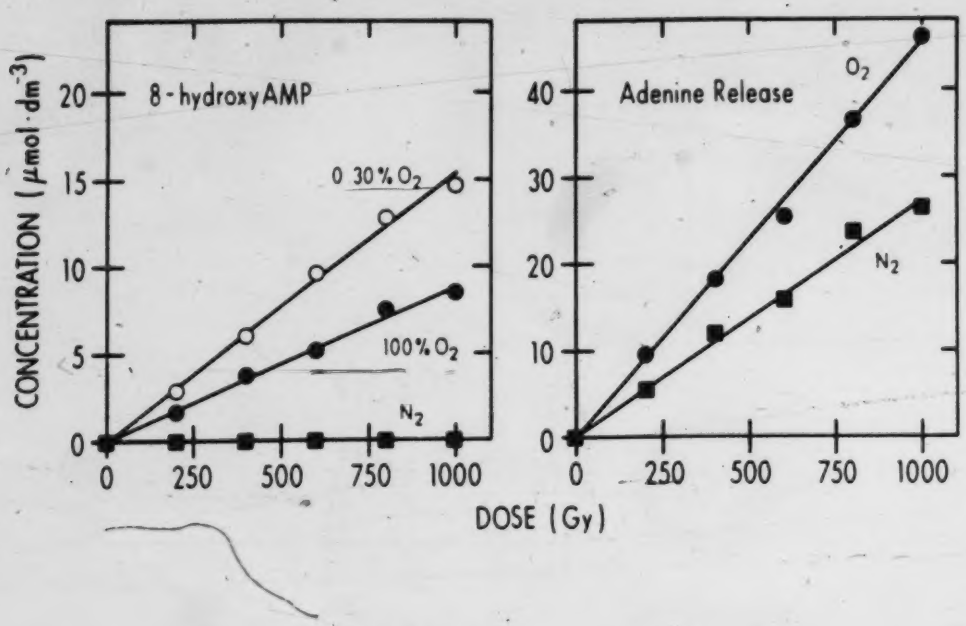

Figure 4: Yield of 8-hydroxyAMP and radiolytic adenine release as a function of dose for various liquid-phase oxygen concentrations. For clarity, dose-yield plots corresponding to 0.15% and 1.62% pO_2 were omitted from 8-hydroxyAMP data and 0.3, 0.15, 1.62% pO_2 from the adenine release data.

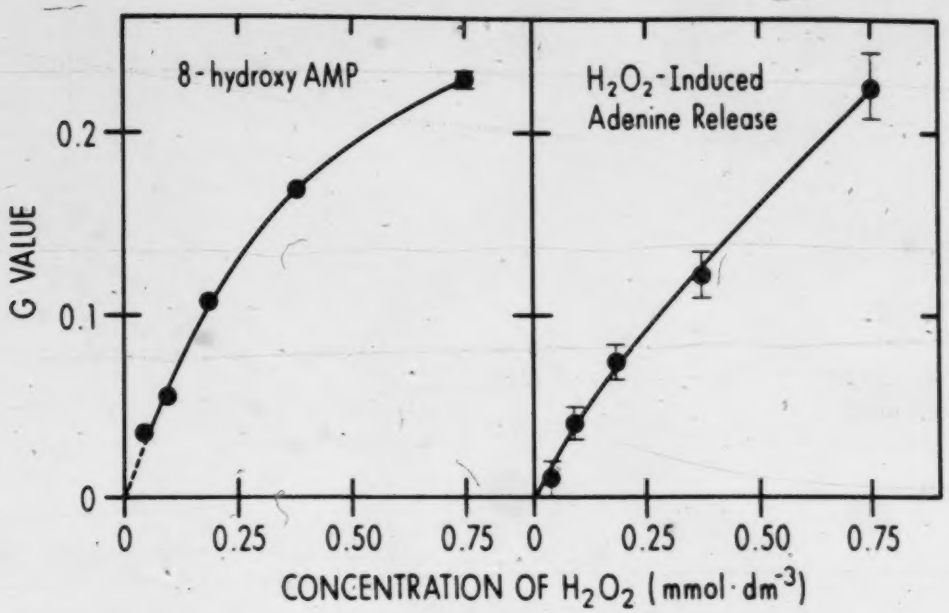

Figure 5: Effect of increasing hydrogen peroxide concentration on product formation in nitrogen-saturated solutions of 5'-AMP (1×10^{-3} mol·dm^{-3}; pH 7.0) irradiated to 1000 Gy. Formation of 8-hydroxyAMP and a hydrogen peroxide-induced component of adenine release increased whereas, 8,5'-cyclonucleotide formation remained constant (data not shown). Each data point represents the mean (± S.E.M.) for three measurements.

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CHAPTER 5

STEREOSELECTIVE INTRAMOLECULAR CYCLIZATION IN IRRADIATED NUCLEIC ACIDS: (R)- AND (S)-8,5'-CYCLOADENOSINE IN POLYADENYLIC ACID

INTRODUCTION

Hydroxyl radical attack can produce either base or sugar phosphate damage in nucleic acids, or hydroxyl radical attack can initiate 8,5'-cyclonucleotide formation (1-4) which represents concomitant sugar phosphate and base damage. The intramolecular cyclization is efficient and leads to both (R)- and (S)-8,5'-cycloadenosine 5'-monophosphates in irradiated adenosine 5'-monophosphate (5). Given that the (R)- and (S)-epimers are not equally good substrates for a variety of adenine nucleotide enzymes (6-8) and that the steric requirements for cyclization are stringent, it was of interest to discover what the distribution of epimers would be in irradiated nucleic acids. The results might have a bearing on the enzymic repair of such molecular lesions in irradiated DNA. Toward this end, we have examined the relative yield of (R)- and (S)-8,5'-cycloadenosine in irradiated polyadenylic acid (poly A) by means of high-performance liquid chromatography (HPLC).

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MATERIALS AND METHODS

Authentic samples of (R)- and (S)-8,5'-cycloadenosine were synthesized according to a published procedure which involved the photolytic cyclization of the 2',3'-O-isopropylidene derivative of 5'-decxy-5'-phenylthioadenosine (9). The identity of the synthesized cyclcadenosine epimers was confirmed by combined gas chromatography - mass spectrometry (M. Dizdaroglu, personal communication). The synthesized compounds were found to be identical to (R)- and (S)-8,5'-cycloadenosine obtained from the radiolysis of adenosine 5'-monophosphate (2,5,7). Adenosine, polyadenylic acid (mol wt. 140-500 kdal) and alkaline phosphatase (Bovine Intestinal Mucosa, Type VII-S) were purchased from the Sigma Chemical Co. (St. Louis, MO). Poly A was irradiated at a concentration of 694 µg/ml in distilled water which had been further purified and deionized by passage through a three module, Barnstead NANOpure water purification system. The pH of the poly A solution was adjusted to pH 7.0 with sodium hydroxide and irradiated in a Gammacell 220 ^{60}Co gamma radiation source (Atomic Energy of Canada Ltd.) to various doses at a dose-rate of 61.4 Gy/min as measured by Fricke dosimetry [$G(Fe^{3+})$ = 15.6]. The solutions were bubbled 15 minutes prior to and, then, throughout the time of irradiation with nitrous oxide which had been deoxygenated by passage through a 0.1 mol·dm^{-3} sodium dithionite trap and then freed of acidic impurities by passage through a 0.1 mol·dm^{-3} sodium carbonate trap. The irradiated poly A was hydrolyzed to the mononucleotide level in 4 mmol·dm^{-3} zinc nitrate

solution (pH 7.0) according to a literature description (10). Hydrolysis of the mononucleotides to the nucleoside level was achieved by alkaline phosphatase treatment (4 units/ml) for 18 hours at 37° in a 0.1 $mol \cdot dm^{-3}$ sodium carbonate/bicarbonate buffer containing 1 $mmol \cdot dm^{-3}$ magnesium chloride (pH 9.6).

HPLC of the hydrolysate was carried out on a Spectra Physics system incorporating a SP8100 pump, SP8110 automatic sample injector, SP8400 variable wavelength detector, and SP4270 computing integrator. A Waters μBondapak C_{18} reversed-phase column (7.8 mm x 30 cm) was used for nucleoside analysis and a Varian MicroPak G3000 SW steric exclusion column (10 mm x 50 cm) was used to follow the chemical hydrolysis of irradiated poly A solutions. The yields of (R)- and (S)-8,5'-cycloadenosine in irradiated solutions of 1.0 $mmol \cdot dm^{-3}$ adenosine (pH 7.0) were determined for comparison with the poly A results.

RESULTS

Chemical hydrolysis of irradiated poly A followed by alkaline phosphatase hydrolysis produced a mixture of ribonucleosides and free adenine which could be separated by reversed-phase chromatography (Figure 1). Quantitation of (R)- and (S)-8,5'-cycloadenosine was achieved by means of standard curves developed with authentic samples. In addition to the epimeric cycloadenosines, adenine and other, as-yet-unidentified radiolysis products were observed. The yields of (R)- and (S)-8,5'-cycloadenosine were linear with dose up to

approximately 400 Gy (Figure 2). A levelling off in the dose-yield plots occurred above this dose in a manner similar to that observed in earlier studies in which an enzyme-linked immunosorbent assay was used to measure the combined yield of (R)- and (S)-8,5'-cycloadenosine *in situ* in irradiated poly A (3). The data in Figure 2 reveal that the (R)-epimer predominates to the extent of 2.5 fold over the (S)-epimer in the dose range up to 400 Gy increasing to 3.5 fold at higher doses. The combined G value (molecules formed for each 100 eV absorbed in the solution) for 8,5'-cycloadenosine in poly A up to a dose of 400 Gy is 0.09. The ratios of (R)- and (S)-8,5'-cycloadenosine for adenosine, adenosine 5'-monophosphate and poly A irradiated to 400 Gy at pH 7.0 under nitrous oxide were 1.8, 0.36 and 2.5 respectively (11).

DISCUSSION

For irradiated adenosine 5'-monophosphate, the relative yields of (R)- and (S)-8,5'-cyclcadenosine 5'-monophosphate are strongly pH dependent (5) with the (S)-epimer predominating at neutral pH. The steric constraints in single-stranded poly A clearly alter the stereoselectivity at neutral pH strongly in favor of the (R)-epimer (Figure 2). An investigation of molecular models of poly A or DNA indicates that the (R)-epimer could, in fact, be expected to predominate in nucleic acids. The gauche-trans conformation about the C(4')-C(5') bond (12) in the adenosine or deoxyadenosine moieties of nucleic acids, which is compatible with intramolecular cyclization

to form the (R)-epimer (Figure 3), predominates in extended polynuclectide chains. The results in Figure 2 indicate that radical formation at C(5') does not greatly change this conformation even in single-stranded poly A where there is the possibility of chain bending about the phosphate ester bond to produce the trans-gauche conformation represented by the (S)-epimer (Figure 3). The lessened flexibility in double-stranded nucleic acids would tend to favor the formation of the (R)-epimer as well where it is known that the yield of 8,5'-cyclodeoxyadenosine in irradiated double-stranded DNA is 2-3 times greater than that in single-stranded DNA (3) which may be in part due to the favorable alignment of base and sugar moieties in the more rigid, double-stranded structure. As far as substrate requirements for adenine nuclectide enzymes such as 5'-nuclectidase are concerned, the (R)-epimer has been found to possess the correct conformation whereas the (S)-epimer does not (7,8). It may be speculated on this limited basis, that the formation of (R)-8,5'-cyclonuclectides in irradiated nucleic acids may not pose a problem for excision by repair enzymes acting at the site of the lesion.

Previous claims for stereospecificity in the formation of 8,5'-cyclonucleosides and -nuclectides (2,4,13) appear now to be incorrect. At best, the process is stereoselective with the stereoselectivity depending very much on the nature of the substrate irradiated. The stereoselectivity in irradiated poly A appears to originate in steric effects imposed by the conformation of the nucleic

acid strand. The source of the stereoselectivities seen for adenosine and adenosine 5'-monophosphate are rather less clear.

Figure 1: Reversed-phase liquid chromatography of hydrolyzed, irradiated poly A on a Waters μBondapak column with an eluent of 10 per cent methanol in 20 mmol·dm^{-3} ammonium formate, pH 4.0. The unmarked peaks are unidentified radiolysis products.

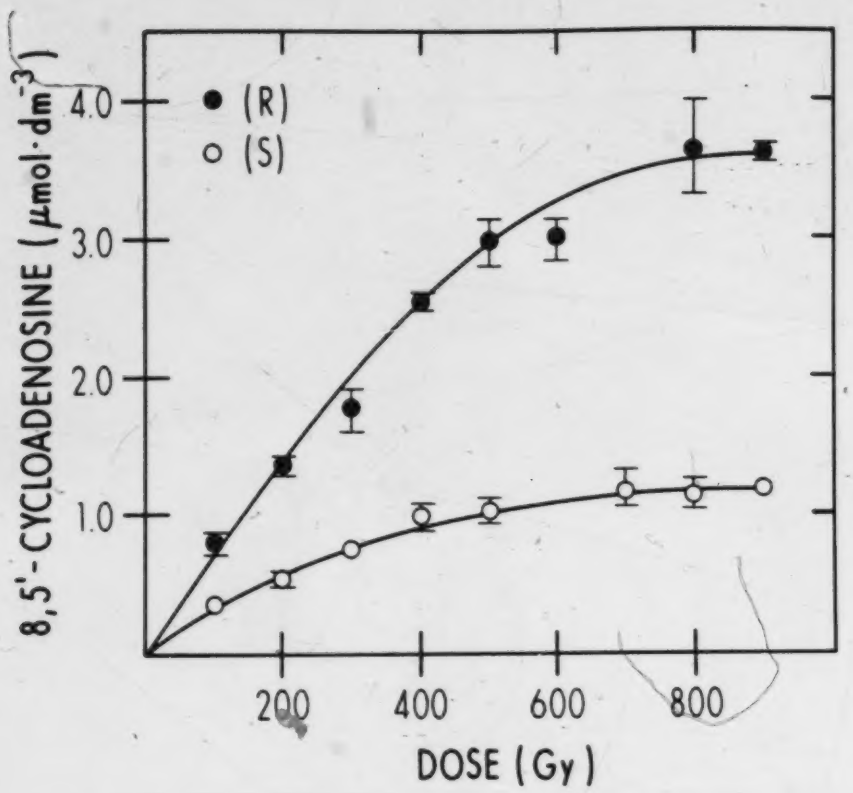

Figure 2: Yields of (R)- and (S)-8,5'-cyclcadenosine in poly A irradiated to various doses of gamma rays at pH 7.0 in nitrous oxide saturated solution. Each point includes the mean and standard deviation from three separate experiments.

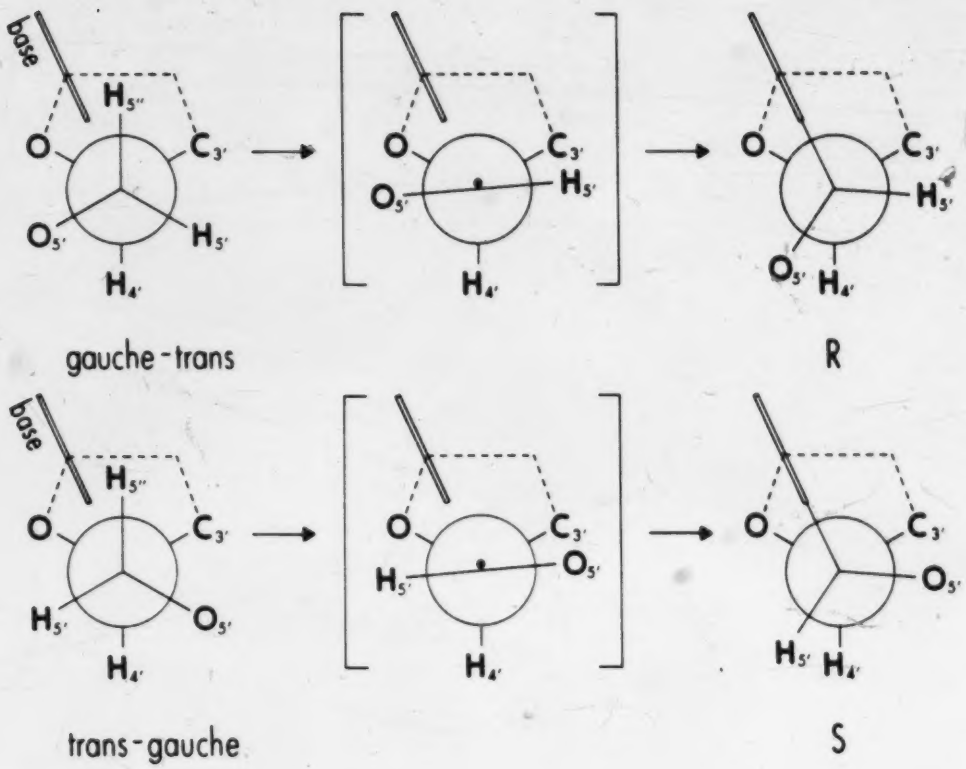

Figure 3: Newman projections showing conformations about the C(4')-C(5') bond which correspond to (R)- and (S)-epimers of 8,5'-cycloadenosine. The projection in brackets is the presumed C(5') free radical intermediate leading to cyclonucleoside formation.

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CHAPTER 6

AN IMMUNOCHEMICAL PROBE FOR 8,5'-CYCLOADENOSINE 5'-MONOPHOSPHATE AND ITS DEOXY ANALOG IN IRRADIATED NUCLEIC ACIDS

INTRODUCTION

A radiation induced intramolecular cyclization of adenosine 5'-monophosphate (5'-AMP), occurring in the absence of oxygen, leads to the formation of 8,5'-cycloadenosine 5'-monophosphate (8,5'-cycloAMP) (2-4). A similar process has also been observed with irradiated solutions of guanosine 5'-monophosphate or its deoxy analog, as well as inosine 5'-monophosphate and deoxyadenosine 5'-monophosphate (2,5). Preliminary reports indicate that intramolecular cyclization of this kind may also occur in irradiated nucleic acids (6,7) and it seems that such modifications might be used as probes for the study of radiation damage in nucleic acids.

Immunochemical assays have considerable potential for the detection of radiation-induced modifications in nucleic acids because of their ability to measure damage with good specificity and sensitivity. In this context, phage neutralization assays (6-9), radioimmunoassays (10,11) and an enzyme-linked immunosorbent assay (12,13) have been used to assess radiation-induced modifications in nucleic acids.

1. A version of this chapter has been published. Fuciarelli, A.F., Miller, G.G. and Raleigh, J.A. An immunochemical probe for 8,5'-cycloadenosine 5'-monophosphate and its deoxy analog in irradiated nucleic acids. Reprinted by permission from Radiat. Res. 104: 272-283, 1985 (Academic Press, Inc.).

In the present report, an enzyme-linked immunosorbent assay (ELISA), with specificity for 8,5'-cycloAMP and its deoxy analog (8,5'-cyclodAMP) is described. Formation of these products are assessed in irradiated solutions of polyadenylic acid (poly A) and DNA saturated with nitrous oxide, nitrogen or oxygen. Preliminary reports of these data have been presented (13-15).

MATERIALS AND METHODS

Reagents

Bovine serum albumin (BSA) Fraction V, Limulus polyphemus hemolymph Type VIII (hemocyanin), poly A (mol. wt. 140-500 kdal), calf thymus DNA, Sigma 104 phosphatase substrate, carbonyldiimidazole, dimethyl sulfoxide (DMSO), tri-n-octylamine, triethylamine and polyoxyethylenesorbitan monolaurate (Tween 20) were purchased from the Sigma Chemical Co. (St. Louis, MO); polyvinyl chloride 96-well microtiter plates were purchased from Dynatech Laboratories Inc. (Chantilly, VA); fetal calf serum, goat serum and Freund's adjuvants were purchased from Gibco Laboratories Inc. (Grand Island, NY); goat anti-rabbit IgG alkaline phosphatase conjugate was purchased from Miles Laboratories Inc. (Naperville, IL) and Spectrapor dialysis membranes (molecular weight cut-off 6,000 - 8,000) were purchased from Spectrum Medical Industries Inc. (Los Angeles, CA). All other reagents were research grade chemicals purchased from local suppliers.

Preparation of Protein-Hapten Conjugates

The (R)- and (S)-epimers of 8,5'-cycloAMP were prepared as

described previously (3,4). The two compounds were greater than 95 per cent pure by high-pressure liquid chromatography with the major impurity in each case being the opposite epimer. No 5'-AMP was detectable in either sample. The protein-hapten conjugates were prepared by a modification of the procedure reported by Johnston et al. (1) using carbonyldiimidazole as the coupling agent. A protein-hapten conjugate prepared by the periodate oxidation method (16) failed to form antibodies with specificity to 8,5'-cycloAMP.

A. Preparation of Hemocyanin- (R)-8,5'-CycloAMP for Immunization

(R)-8,5'-cycloAMP (13.8 mg) was dried *in vacuo* with three consecutive additions and evaporations of dry pyridine followed by dry benzene. The residue was dissolved in a mixture of dry DMSO (2.0 ml), triethylamine (0.1 ml) and tri-*n*-octylamine (0.05 ml). Carbonyldiimidazole (30 mg) was added, and the reaction mixture was stirred for 60 min at room temperature. This solution was added to a solution of NaI (57.3 mg) in 5.0 ml of dry acetone. The resultant white precipitate was vortexed, pelleted by centrifugation, and washed twice with dry acetone. The imidazolide of (R)-8,5'-cycloAMP was evaporated to dryness and then dried over P_2O_5 for 2 hr before further reaction. The yield of the imidazolide derivative of (R)-8,5'-cycloAMP was 58% (10.2 mg).

A solution of hemocyanin (50.0 mg) in dry DMSO (4.0 ml) was added to the imidazolide derivative of (R)-8,5'-cycloAMP and stirred for 60 min in a 70°C water bath. The solution was then placed in a dialysis membrane and extensively dialyzed with 0.14 $mol \cdot dm^{-3}$

NaCl, 1 mmol·dm^{-3} KH_2PO_4, 8 mmol·dm^{-3} Na_2HPO_4, 3 mmol·dm^{-3} KCl, pH 7.4 (PBS). The protein-hapten conjugate was obtained as a precipitate and suspended in 10.0 ml of PBS. Aliquots of this mixture (0.5 ml) were dispensed into tubes and stored frozen at -17°C.

B. Preparation of BSA- (S)-8,5'-CycloAMP for ELISA

The imidazolide derivative of (S)-8,5'-cycloAMP was prepared from (S)-8,5'-cycloAMP (36.0 mg) and conjugated to BSA (50.8 mg) by methods similar to those for the conjugation of the imidazolide derivative of (R)-8,5'-cycloAMP to hemocyanin as described above. The yield of the imidazolide derivative of the 8,5'-cycloAMP intermediate was 61%. Two moles of (S)-8,5'-cycloAMP were bound per mole of BSA, as estimated by spectrophotometry (15).

Immunization and Treatment of Antisera

Two New Zealand white female rabbits were injected with the hemocyanin-(R)-8,5'-cycloAMP conjugate. Prior to the initial immunization, 30 ml of blood were collected from each rabbit by cardiac puncture. These preimmune serum samples were used as control sera in subsequent assays. Each rabbit received a total of 0.5 ml of the antigen (6 mg/ml), emulsified with an equal volume of Freund's complete adjuvant, injected at multiple subscapular sites. Similar booster injections, emulsified in Freund's incomplete adjuvant, were administered by the same route on days 21 and 26. Sera (30 ml) were collected by cardiac puncture on days 21, 43, 60 and 89.

Immediately following collection of blood by cardiac puncture, the blood was allowed to clot, the supernatant was drawn off and

the serum was recentrifuged thrice. Aliquots of 2.0 ml were stored at -17°C. Repeated thawing and refreezing were avoided.

ELISA Methodology

A. Reagent Dilution Assay

The reagent dilution assay was carried out in order to determine the titer of the antiserum, the optimal dilution of conjugate used to coat the wells of the microtiter plates, and the dilution at which an ELISA reading of 50% maximum value occurs. Negative controls incorporated into each microtiter plate for this screening assay and competitive inhibition assays consisted of wells containing: 1) no protein-hapten conjugate; 2) 0.5 mg/ml BSA and 3) 10^{-2} dilutions of preimmune serum. The remaining wells on the microtiter plate were coated with BSA-(S)-8,5'-cycloAMP by means of treatment with 0.2 ml aliquots of the conjugate, serially diluted in coating buffer (15 mmol·dm^{-3} Na_2CO_3, 35 mmol·dm^{-3} $NaHCO_3$, pH 9.6), for 14 hr at 4°C. The wells of the plates were each washed five times with PBS, 0.05% Tween 20, 3 mmol·dm^{-3} NaN_3 (PBS-Tween) with a plastic wash bottle. To prevent non-specific binding to the wells of the goat anti-rabbit IgG alkaline phosphatase conjugated antiserum, each well was exposed to 0.2 ml of 1% goat serum in PBS-Tween. Serum obtained from the same rabbit before and after immunization (day 60) was serially diluted in PBS-Tween and 0.1 ml of the diluted serum was added to each well. After a 2 hr incubation at 37°C, the plates were washed five time with PBS-Tween and incubated with 0.1 ml of goat anti-rabbit IgG alkaline phosphatase conjugate per well

(1:400 dilution in PBS-Tween supplemented with 1% fetal calf serum to prevent nonspecific binding). The plates were washed five more times with PBS-Tween and then twice with 10% diethanolamine buffer (pH 9.8) before adding to each well 0.1 ml of Sigma 104 phosphatase substrate (0.1 mg/ml) diluted in 10% diethanolamine buffer (pH 9.8). The enzymatic reaction was allowed to continue for 30-60 minutes at which time 0.05 ml of 3.0 N NaOH was added to each well in order to inhibit the enzyme reaction. The extent of colour development from the phosphatase substrate was measured using a Dynatech MR600 Microplate Reader (Dynatech Laboratories Inc.) using a reference wavelength of 610 nm and an operating wavelength of 410 nm. The spectrophotometer was zeroed on an uncoated well containing a 10^{-2} dilution of preimmune rabbit serum diluted in PBS-Tween.

B. Competitive Inhibition Assay

For inhibition studies, the dilution of antiserum corresponding to 50% of the maximal ELISA reading from the reagent dilution assay was selected. In a representative experiment, a serial dilution of a potential inhibitor was prepared. A 0.18 ml aliquot of each concentration of inhibitor was added to 0.020 ml of antiserum which had been diluted tenfold. Preimmune serum was adjusted to a final dilution corresponding to that of the test antiserum. These solutions were incubated for 2 hr at 37°C prior to being added to the microtiter plates. The microtiter plates were exposed to these solutions for 2 hr at 37°C, and subsequently washed from the wells with five changes of PBS-Tween. Addition of the anti-rabbit alkaline phosphatase second

antibody and subsequent exposure of the enzyme substrate to the wells was as described above.

For radiation chemical studies, the concentration of 8,5'-cycloAMP (or its -deoxy analog) was determined from calibration curves using (S)-8,5'-cycloAMP developed on the same microtiter plate. If 8,5'-cyclonucleotide formation in the irradiated nucleic acids included both (R)- and (S)-epimers, then the yield as measured using the (S)-epimer as the competitive inhibitor would overestimate the yield of 8,5'-cyclonucleotides. The question of whether 8,5'-cyclonucleotides are formed stereoselectively in irradiated nucleic acids is presently under investigation. The binding of the antibodies to the hapten was assumed to be equivalent whether the the hapten was in free solution or present as an integral part of the polynucleotide. While this assumption would seem suitable for the purposes of the present study, it would be inappropriate to consider the yields of 8,5'-cyclonucleotide expressed in Figures 3, 4 and 5 as anything but relative yields as is discussed below.

Irradiation of Poly A and DNA

Samples of poly A or DNA were bubbled with nitrous oxide, nitrogen or oxygen for 15 minutes prior to and then throughout the time of irradiation. Nitrous oxide and nitrogen were scrubbed free of oxygen by passage through a 0.1 $mol \cdot dm^{-3}$ sodium dithionite trap and scrubbed free of acidic impurities by passage through a 0.1 $mol \cdot dm^{-3}$ sodium carbonate trap. Oxygen was passed through the latter trap only.

Irradiation was performed in a Gammacell-220 ^{60}Co gamma radiation source (Atomic Energy of Canada Ltd.) at a dose of 78.9 Gy/min as

determined by Fricke dosimetry [$G(Fe^{3+}) = 15.6$, (17)].

Poly A (365 µg/ml) was dissolved in 100 mmol·dm^{-3} phosphate buffer (pH 7.0). A series of solutions was saturated with N_2O, N_2 or O_2 and irradiated to various doses. Each sample was diluted tenfold in PBS-Tween prior to ELISA to prevent nonspecific crossreactivity.

Calf thymus DNA (373 µg/ml) was dissolved in 100 mmol·dm^{-3} phosphate buffer (pH 7.0). Heat-denatured DNA was prepared by heating 20 ml of the stock DNA solution to 95°C for 5 minutes followed by rapid cooling on ice (11). A series of native and heat-denatured solutions of DNA was bubbled, irradiated and diluted in PBS-Tween as described for the poly A samples. Aliquots of the diluted, irradiated native DNA samples were also heat-denatured by the procedure described.

Effect of Dimethyl Sulfoxide on the Yield of 8,5'-cycloAMP from Irradiated Poly A

Solutions of DMSO were prepared containing poly A (357 µg/ml) in 100 mmol·dm^{-3} phosphate buffer (pH 7.0). The solutions were saturated with either nitrous oxide or nitrogen, irradiated to 100 Gy and diluted tenfold in PBS-Tween prior to ELISA.

RESULTS

A sample of antiserum from one of the rabbits was screened using the BSA-(S)-8,5'-cycloAMP conjugate to coat the wells of the microtiter plates. A thousand-fold dilution of the conjugate containing 10 µg/ml protein-hapten provided optimal adsorption (Figure 1). Reducing the concentration of the conjugate during the adsorption

process below 10 µg/ml significantly reduced the maximum ELISA value. Wells coated with 0.5 mg/ml BSA exhibited no significant cross-reactivity with the antiserum. The titer of the antiserum was 10^{-3} and did not change after repeated immunization. A 50% positive response occurred at a one hundred-fold dilution of antiserum and competitive inhibition studies were performed at this dilution.

Specificity of the Antiserum

The concentration of various nucleosides, nucleotides and bases required to inhibit the binding of the antiserum to immobilized BSA-(S)-8,5'-cycloAMP by 50% was determined (Figure 2). Table I compares the specificity of the antiserum by listing the IC_{50} - the concentration ($\mu mol \cdot dm^{-3}$) required for 50% inhibition of the antiserum binding (18) - as calculated from Figure 2. Competition for the anti-hemocyanin-(R)-8,5'-cycloAMP antibodies was most effective with the immunizing hapten-(R)-8,5'-cycloAMP. The concentration required for 50% inhibition (IC_{50}) for the (R)-epimer was 28 $nmol \cdot dm^{-3}$ and 250 $nmol \cdot dm^{-3}$ for the (S)-epimer. The contribution of the ribose 2'-OH to the specificity of the antiserum appears minimal as determined from the similar IC_{50} for 5'-AMP (19 $\mu mol \cdot dm^{-3}$) and for 5'-dAMP (10 $\mu mol \cdot dm^{-3}$). However, the position of the phosphate group is significant as demonstrated by the observations that the IC_{50} for 3'-AMP and adenosine were 100 times greater than that for 5'-AMP. Loss of both the ribose 2'-OH group and the phosphate group, as exemplified by 2'-deoxyadenosine, resulted in a significant increase

in the IC_{50} to 3500 $\mu mol \cdot dm^{-3}$. Adenine exhibited an IC_{50} of 550 $\mu mol \cdot dm^{-3}$ which is unexpectedly low considering the low specificity of the antiserum to 2'-deoxyadenosine. Cross-reactivity of the antiserum with 5'-GMP, guanosine and guanine, is insignificant. The IC_{50} for 3'-GMP is 420 $\mu mol \cdot dm^{-3}$, which is unexpectedly low for a compound containing both guanine as the base and a 3'-phosphate group.

Irradiation of Poly A

The relative yield of 8,5'-cycloAMP in irradiated solutions of poly A in 100 $mmol \cdot dm^{-3}$ phosphate buffer (pH 7.0) in nitrous oxide- or nitrogen-saturated solutions is a linear function of the dose below 200 Gy but drops off at higher doses (Figure 3). The ratios of the yields of 8,5'-cycloAMP from poly A irradiated under nitrous oxide to those under nitrogen vary from 1.21 at 50 Gy to 1.35 at 500 Gy (Figure 3). The formation of 8,5'-cycloAMP was strongly inhibited in oxygen-saturated solutions.

Hydroxyl Radical and 8,5'-cyclonucleotide Formation in Poly A

To test the contribution of the hydroxyl radical ($^{\cdot}OH$) to 8,5'-cyclonucleotide formation in poly A, the $^{\cdot}OH$ scavenger, DMSO [$k_{DMSO + {}^{\cdot}OH} = 6 \times 10^9 \; dm^3 \cdot mol^{-1} \cdot s^{-1}$, (19)] was used. At concentrations of DMSO greater than 10^{-6} $mol \cdot dm^{-3}$, following a radiation dose of 100 Gy, a decrease in the yield of 8,5'-cycloAMP occurred (Figure 4). From these data a reaction rate constant of $1.3 \times 10^8 \; dm^3 \cdot mol^{-1} \cdot s^{-1}$ per nucleotide can be estimated. This value is lower than the overall reaction rate constant per nucleotide for poly A of $3.8 \times 10^8 \; dm^3 \cdot mol^{-1} \cdot s^{-1}$ as determined by competition

kinetics (20) and a value of 9×10^8 $dm^3 \cdot mol^{-1} \cdot s^{-1}$ determined for the rate of hydroxyl radical reaction with the bases of poly A (21), but perhaps not unreasonable for a reaction occurring at one site, the 5'-carbon on the phosphodiester backbone.

Irradiation of DNA

The effect of the structure of the DNA during ELISA was investigated to establish if the detectability of 8,5'-cyclodAMP could be increased by heat-denaturation. Native DNA was irradiated then assayed in the native or heat-denatured form. In general, the apparent yield of 8,5'-cyclodAMP was greater if the assay was performed on heat-denatured DNA (Figure 5). For example, at 600 Gy the yield of 8,5'-cyclodAMP in irradiated, nitrous oxide-saturated solutions of native DNA, assayed in the native form, was 60 per cent of its yield when the DNA was heat-denatured prior to ELISA (compare Fig 5A and 5B).

To compare the effect of DNA structure on the yield of 8,5'-cyclodAMP, native or heat-denatured DNA was irradiated then, in each case, assayed as heat-denatured DNA. The yield of 8,5'-cyclodAMP in irradiated, native DNA was higher than that in irradiated, heat-denatured DNA (Figure 5). In nitrous oxide-saturated solutions irradiated to 600 Gy, the amount of 8,5'-cyclodAMP detected in native DNA solutions (Figure 5B), was 2.8 times the amount detected in DNA which had been heat-denatured before irradiation (Figure 5C). Similar results were observed for irradiation of nitrogen-saturated solutions.

For DNA irradiated in the native form and heat-denatured for

assay, the ratios of the yield of 8,5'-cyclodAMP under nitrous oxide compared to those under nitrogen vary from 1.0 at 200 Gy to 1.6 at 800 Gy (Figure 5B). For DNA irradiated and assayed in the heat-denatured form, the N_2O/N_2 ratios vary from 1.2 at 200 Gy to 2.4 at 800 Gy (Figure 5C). The yield of 8,5'-cyclodAMP in oxygen-saturated solutions of native DNA is barely detectable (data not shown) which is consistent with the observation that oxygen inhibits cyclization in poly A (this work) and in mononucleotides (3).

DISCUSSION

Immunochemical techniques provide a sensitive and specific method for probing structural and functional properties in nucleic acids (see Munns and Liszewski (22) for review). In particular, they have been used to identify DNA-carcinogen adducts (see Müller and Rajewsky (23) for review) and radiation-induced modifications to nucleic acids. In this latter context, immunochemical assays have been developed with specificity to nucleic acid base damage as exemplified by thymine glycols (10,24), 5-hydroxymethyldeoxyuridine (7-9) and 8-hydroxyadenine (11) and show considerable promise with respect to the study of radiation-induced modifications in nucleic acids.

Radiation chemical studies with purine mononucleotides have revealed that an intramolecular cyclization, initiated by $^{\cdot}OH$ attack on the C(5') carbon, can occur resulting in the formation of 8,5'-cyclonucleotides (2-4). Preliminary results indicated that cyclonucleotide formation also occurred in irradiated nucleic acids

(6,7) and could be useful as a novel probe for radiation damage. For example, the formation of 8,5'-cyclonucleotides may be important in double-stranded nucleic acids where the phosphodiester backbone is exposed to, and the bases shielded from, direct radical attack. Moreover, intramolecular cyclization in nucleic acids may be influenced by hydrogen bonding and base stacking relationships in the macromolecules, since formation of the C(8)-C(5') bond requires correct alignment of the base and sugar moieties. Another interesting feature of 8,5'-cyclonuclectides arises from enzymatic studies which reveal a strong substrate specificity with respect to the two epimeric forms (R and S) of this product (25-29). This raises the possibility that repair enzymes may show a similar substrate specificity with respect to the repair of 8,5'-cyclonucleotide formation within irradiated nucleic acids. As a first step in a study of the significance of 8,5'-cyclonuclectides, we have utilized immunochemical techniques to investigate formation of this species in irradiated nucleic acids.

The specificity of the ELISA technique for 8,5'-cyclonucleotide detection is demonstrated by the competitive inhibition studies which further indicate that the polyclonal antibodies which recognize 8,5'-cycloAMP should also recognize the corresponding deoxyribonucleotide with approximately the same affinity. For example, the 2'-OH group was found not to be a major determinant as shown by the fact that the affinity of the antiserum for 5'-AMP and 5'-dAMP is similar (Figure 2, Table I). In contrast, the phosphate group

(compare the affinity of 5'-AMP with that of adenosine) and the adenine base (compare the affinity of 5'-AMP with that of 5'-GMP) are major determinants recognized by the antiserum. Taken together, the data contained in Figure 1 suggest that the major antigenic determinants recognized by the antiserum exist in the unique structure relationship between the adenine base, phosphate group and sugar moiety which results from the constraints imposed by the presence of the C(8)-C(5') intramolecular bond in 8,5'-cycloAMP. Having said this, we do not, at present have an independent way of demonstrating that the 8,5'-cyclonucleotide detected by the ELISA assay in irradiated nucleic acids is in fact due primarily to this structure as has been possible with thymine glycol (12). However, we believe that the indirect evidence in favor of 8,5'-cyclonucleotide formation in irradiated nucleic acids is compelling. For example, the apparent ability of the polyclonal antibodies raised to the (R)-8,5'-cycloAMP hapten to distinguish between (R)- and (S)-epimers (Figure 2) argues strongly in favor of a narrow structural specificity for the antibodies. It is true that the antibodies recognize determinants in unirradiated 5'-AMP and 5'-dAMP but under the conditions of nucleic acid dilution in our assay, antibody binding to unirradiated nucleic acids cannot be detected. The fact that the DMSO protection against 8,5'-cyclonucleotide formation in both poly A and DNA shows a first order dependence on DMSO concentration is consistent with antibody binding to $^{\cdot}OH$-initiated damage in the nucleic acids and not to undamaged nucleotide constituents. Furthermore, irradiation under

oxygen completely inhibits antibody binding to irradiated nucleic acids (Figure 3) which fits in with previous observations from the mononucleotide studies (3) and indicates that oxygen effectively traps the nucleotide radical which, in the absence of oxygen, undergoes intramolecular cyclization.

While there is no apparent reason to believe that the polyclonal antibodies were binding to anything other than 8,5'-cyclonucleotides, the absence of an independent assay does limit the assay to one of measuring relative rather than absolute yields of radical damage in nucleic acids. The absolute quantitation of radical damage in nucleic acids by ELISA is further complicated by an uncertainty with respect to the relative reactivity of the polyclonal antibodies to the hapten in free solution and in polymeric nucleic acids (11,30). For example, antibodies raised against carcinogen-modified nucleosides generally exhibit a higher affinity for the free hapten than for haptens incorporated in single- or double-stranded DNA (23,30). In contrast, it is reported that an antiserum specific for thymine glycol recognizes the modified base to a greater extent in DNA than in the corresponding mononucleotide (12). For these reasons the yields expressed in Figures 3-5 cannot at this time be considered absolute measures of 8,5'-cyclonucleotide formation in the nucleic acids and therefore cannot be used to calculate G values. Only in favored situations such as thymine glycol (10,12) or 8-hydroxyadenosine (11) where independent quantitation is attainable will ELISA or radioimmunoassay be quantitative in an absolute sense. However, for many studies involving, for example, the effect of

radiation modifiers on nucleic acid damage, measurement of relative yields would be very useful.

If it is accepted that the structural determinant for the polyclonal antiserum includes a strong component from the C(8)-C(5') bond between adenine and deoxyribose moieties, it is perhaps not surprising that the apparent yield of the 8,5'-cyclonucleotide is somewhat higher if the ELISA is performed after heat-denaturation of the irradiated DNA (Figure 5) since the C(8)-C(5') region will be partially buried in double-stranded DNA. Somewhat more remarkable is the observation that the yield of the 8,5'-cyclonucleotide is higher when DNA is irradiated in the double-stranded as compared to the single-stranded form (Figure 5). This fact makes it clear that any restriction of molecular motion within the adenine mononucleotide moiety which might occur upon its incorporation in DNA does not prevent cyclonucleotide formation. There is the distinct possibility that a more favorable spatial orientation of adenine and deoxyribose moieties, with respect to cyclonucleotide formation, resides in double-stranded DNA than in single-stranded DNA. One possible explanation for reduced yield of the 8,5'-cyclonucleotide in irradiated single-stranded DNA as compared to double-stranded DNA may be that the *syn* conformation may occur more often in single-stranded DNA thereby reducing the probability of forming the C(8)-C(5') intramolecular bond. Alternatively, the relative inaccessibility of DNA bases to direct $^{\cdot}OH$ attack in double-stranded DNA, as invoked in studies of base damage (31,32), may lead to a greater proportion of $^{\cdot}OH$ attack at the phosphodiester backbone

and hence an increased yield of 8,5'-cyclonucleotides in double-stranded DNA.

From earlier studies with 5'-AMP it was anticipated that the yield of the 8,5'-cyclonucleotide would be increased in nitrous oxide-saturated solution of the irradiated nucleic acids. Nitrous oxide converts hydrated electrons to $^{\cdot}OH$ radicals [$N_2O + e^-_{aq} \xrightarrow{H_2O} {}^{\cdot}OH + OH^- + N_2$] (33) thereby effectively doubling the yield of $^{\cdot}OH$ radicals relative to nitrogen-saturated solutions. In the case of 5'-AMP, the G value for the formation of 8,5'-cycloAMP was found to be approximately doubled in nitrous oxide-saturated solution (3). The result in the case of both poly A and DNA appears to be more complex even though the fact that DMSO protects against cyclonucleotide formation in poly A (Figure 4) and DNA (data not shown) is consistent with $^{\cdot}OH$ radicals being the initiating species in the nucleic acids as it is for 5'-AMP (3). For example, the presence of nitrous oxide does not lead to the expected enhancement in 8,5'-cycloAMP yield in irradiated poly A and the increase which does appear (a maximum N_2O/N_2 factor of 1.3 at 500 Gy) is dose-dependent. A similar dose-dependency is observed for DNA (Figure 5) although at the highest doses used for DNA (800 Gy) the yield under nitrous oxide is near the expected factor of two compared to that under nitrogen. It is known that hydrated electrons react with 8,5'-cycloAMP to cleave the phosphate ester bond (34) and the relatively small, dose-dependent effect of nitrous oxide, seen for poly A and DNA up to doses of 500 Gy, may be accounted for more in terms of electron scavenging than a higher yield of $^{\cdot}OH$ radical

as such. Whether the increasing divergence between yields under nitrous oxide and nitrogen for DNA with increasing doses above 500 Gy could be acccunted for by a single such mechanism is not known but is presently under investigation.

In summary, an immunochemical assay for 8,5'-cyclo(d)AMP has been developed and appears to be suitable as a probe for radiation damage in nucleic acids. The yield of 8,5'-cyclodAMP was found to be two to three times higher for DNA irradiated in the double-stranded, as distinct from the single-stranded, form. The ·OH radical appears to initiate 8,5'-cyclonuclectide formation in the nucleic acids but irradiation under nitrous oxide, particularly at lower doses, does not lead to the expected increases in the yield of this product. Oxygen inhibits the formation of 8,5'-cyclonuclectides in irradiated nucleic acids indicating that oxygen effectively traps the nuclectide radical which, in the absence of oxygen, undergoes intramolecular cyclization.

TABLE I: REACTIVITY OF ANTI-HEMOCYANIN-(R)-8,5'-CYCLOAMP ANTISERUM

HAPTEN: (R)-8,5'-CYCLOAMP

INHIBITOR	IC_{50} [a]	INHIBITOR	IC_{50}
(R)-8,5'-cyclcAMP	0.028	2'-dAdo	3500
(S)-8,5'-cyclcAMP	0.250	Ade	550
5'-AMP	19	5'-GMP	3500
5'-dAMP	10	3'-GMP	420
3'-AMP	190	Guo	7000
Ado	190	Gua	7000

[a] IC_{50} IS THE CONCENTRATION ($\mu mol \cdot dm^{-3}$) REQUIRED FOR 50% INHIBITION OF THE ANTISERUM BINDING

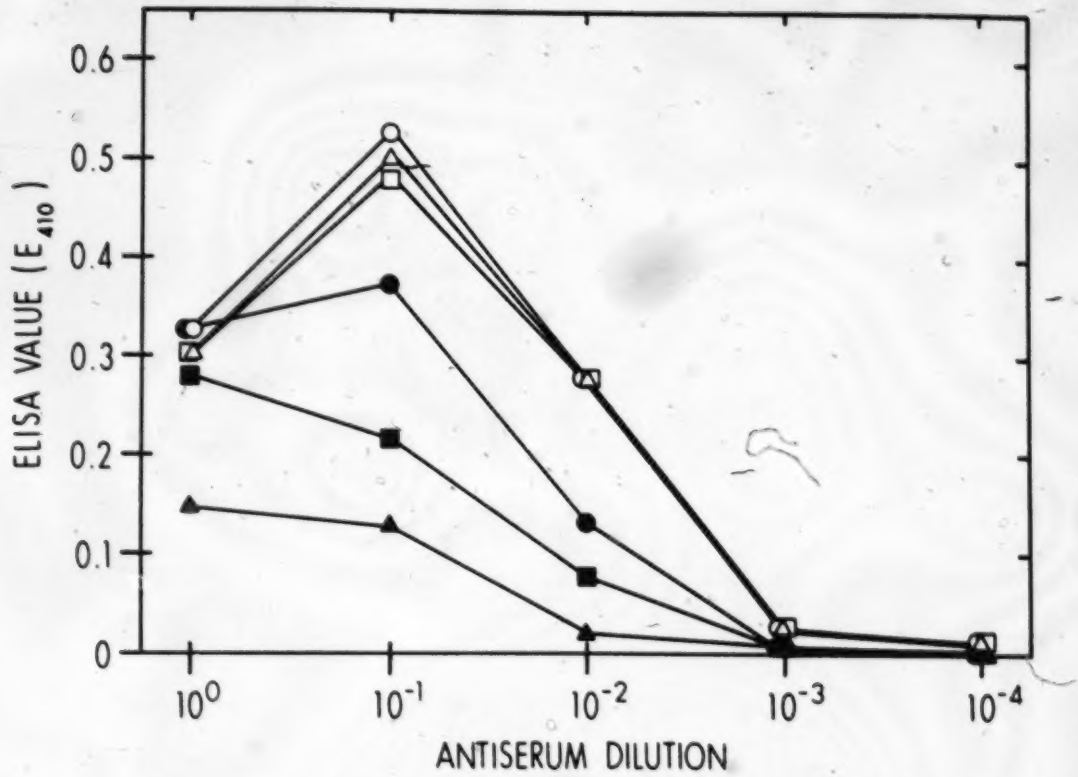

Figure 1: Reagent dilution assay for anti-hemocyanin- (R) 8,5'-cycloAMP antiserum. Antiserum containing anti-hemocyanin-(R)- 8,5'-cycloAMP antibodies was screened with microtiter plates coated with BSA-(S)-8,5'-cycloAMP in an ELISA. Dilution of conjugate in coating buffer: 10^{-1} △, 10^{-2} □, 10^{-3} ○, 10^{-4} ●, 10^{-5} ■ and 10^{-6} ▲.

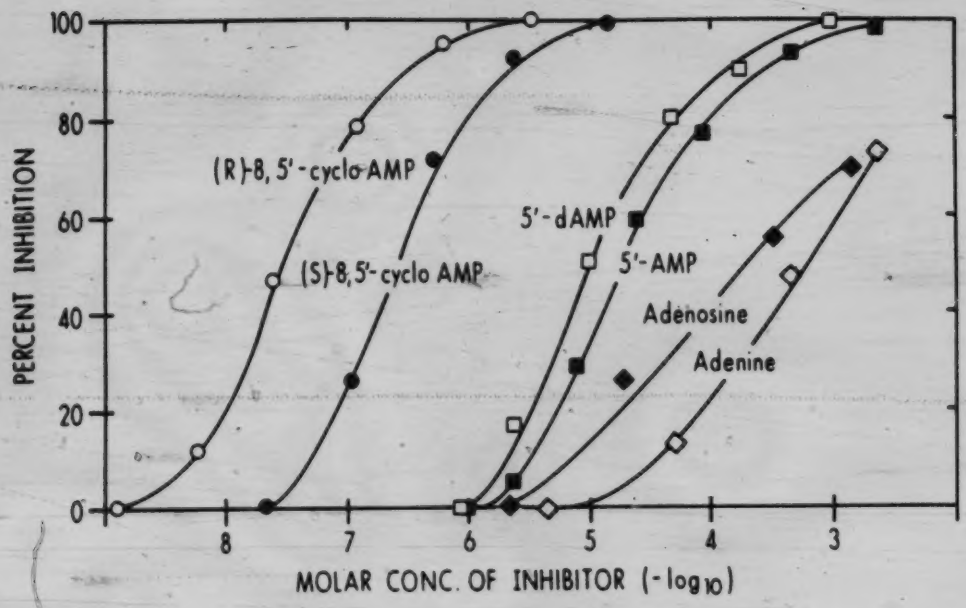

Figure 2: Competitive inhibition curves for anti-hemocyanin-(R) 8,5'-cycloAMP antiserum. Dilutions of various compounds were preincubated with antiserum at a final dilution of 1×10^{-2}, then added to microtiter wells precoated with 10 µg/ml of BSA-(S)-8,5'-cycloAMP for competitive ELISA. Immunization was performed with a hemocyanin-(R) 8,5'-cycloAMP conjugate.

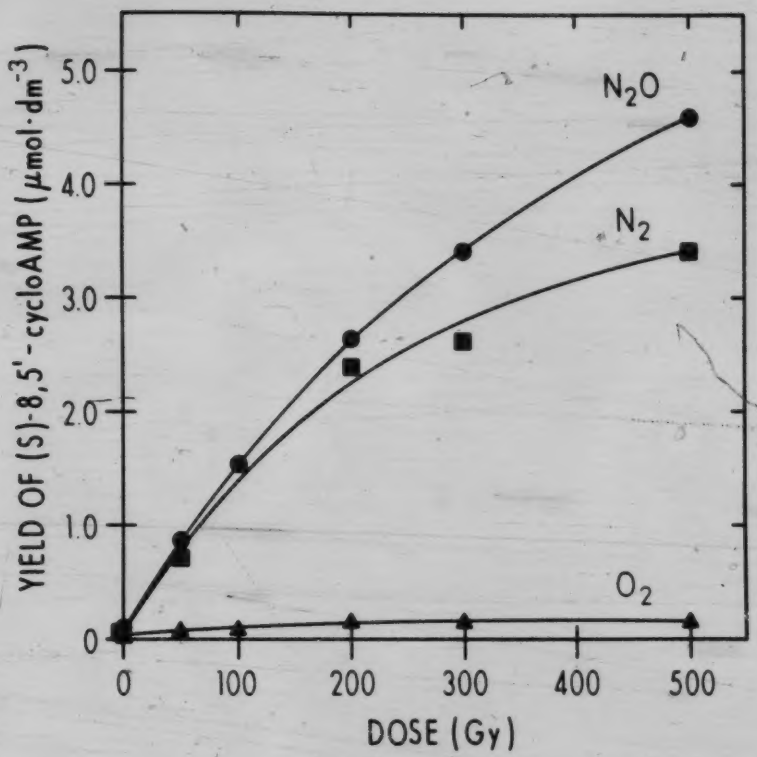

Figure 3: Yield of 8,5'-cycloAMP from irradiated solutions of poly A (365 μg/ml) in 100 mmol·dm^{-3} phosphate buffer bubbled with N_2O (●), N_2 (■) or O_2 (▲). Each data point represents the average of two independent irradiations assayed on the same ELISA plate. The average range of the data is less than 5%. As discussed in the text, the yields of 8,5'-cycloAMP are derived from competitive ELISA and are not absolute measures of 8,5'-cycloAMP in poly A.

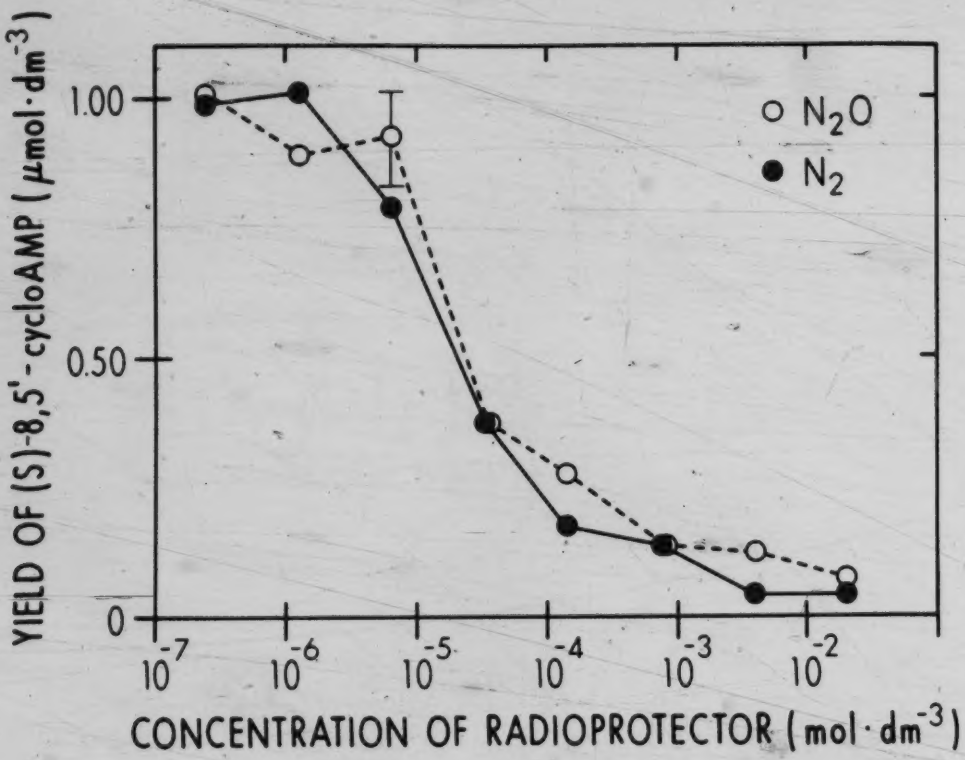

Figure 4: Effect of increasing DMSQ concentration on the yield of 8,5'-cycloAMP from polyadenylic acid (357 μg/ml) irradiated under nitrous oxide (○) or nitrogen (●) to 100 Gy at pH 7.0 in 100 mmol·dm^{-3} phosphate buffer. Each data point represents the mean of 3 measurements and the error bars illustrated represent the greatest standard error for the experiment.

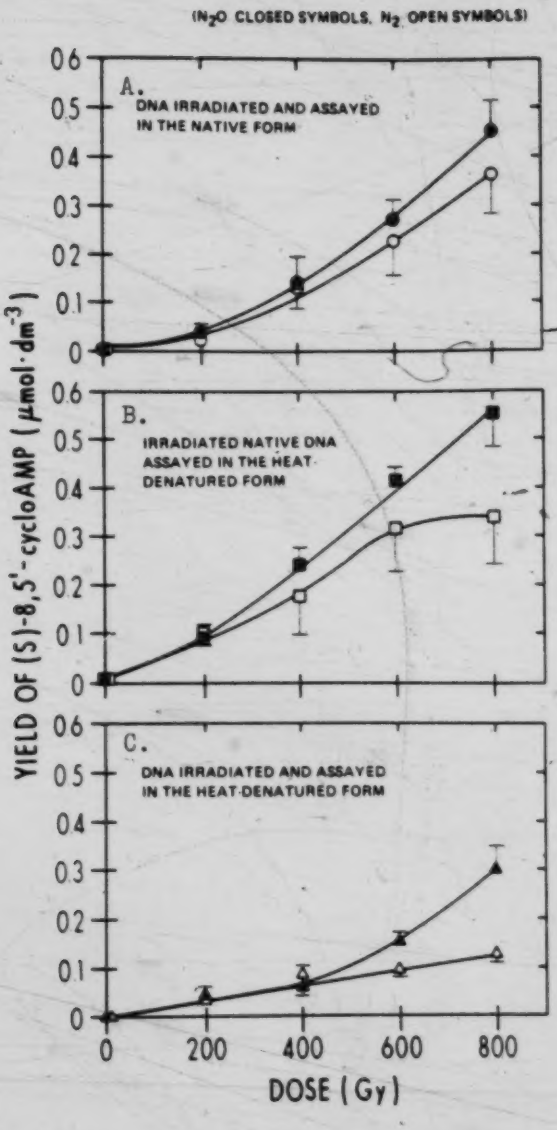
(N_2O CLOSED SYMBOLS, N_2 OPEN SYMBOLS)
A.
DNA IRRADIATED AND ASSAYED IN THE NATIVE FORM
B.
IRRADIATED NATIVE DNA ASSAYED IN THE HEAT-DENATURED FORM
C.
DNA IRRADIATED AND ASSAYED IN THE HEAT-DENATURED FORM
YIELD OF (5)-8,5'-cycloAMP (μmol·dm⁻³)
0 0.1 0.2 0.3 0.4 0.5 0.6
0 200 400 600 800
DOSE (Gy)

Figure 5: The yield of 8,5'-cyclodAMP from solutions of DNA (373 µg/ml) irradiated under nitrous oxide (closed symbols) or nitrogen (open symbols). A. DNA irradiated and assayed in the native from. B. Irradiated native DNA assayed in the heat-denatured form. C. DNA irradiated and assayed in the heat-denatured form. Each data point represents the average of six ELISA measurements ± S.E.M.

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CHAPTER 7

INTRAMOLECULAR CYCLIZATION IN IRRADIATED NUCLEIC ACIDS. CORRELATION BETWEEN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND AN IMMUNOCHEMICAL ASSAY FOR 8,5'-CYCLOADENOSINE IN IRRADIATED POLY A.

INTRODUCTION

Strong oxidizing agents such as the hydroxyl radical ('OH) can damage nucleic acids at both base and sugar phosphate moieties. A variety of products arising from direct 'OH attack on nucleic acid bases have been identified (1,2). Strand breaks and base release are events originating from 'OH attack at the sugar phosphate backbone (3,4). Quantitative studies of the distribution of 'OH attack on adenine nucleotides (5) show that 8,5'-cyclonucleotide formation, a type of damage incorporating both sugar phosphate and base damage (5-8), is also a significant process. This type of base damage might be important in double-stranded nucleic acids where the bases are at least partially shielded from direct oxidative damage (9,10). With this in mind we developed an enzyme-linked immunosorbent assay (ELISA) with specificity for 8,5'-cyclonucleotide formation and have demonstrated that this type of intramolecular cyclization occurs in irradiated nucleic acids (11). Competitive ELISA indicated that

1. A version of this chapter has been accepted for publication. Fuciarelli, A.F., Shum, F.Y. and Raleigh, J.A. Intramolecular cyclization in irradiated nucleic acids. Correlation between high-performance liquid chromatography and an immunochemical assay for 8,5'-cycloadenosine in irradiated poly A. Reprinted by permission from Radiat. Res. 110: 000-000, 1987 (Academic Press Inc.).

the formation of the 8,5'-cyclonucleotide occurred in both polyadenylic acid (poly A) and DNA (11). However, under these conditions the immunochemical technique is not strictly quantitative. We have now compared the yield of 8,5'-cycloadenosine formation in irradiated poly A as measured by both high-performance liquid chromatography (HPLC) and ELISA techniques. The HPLC study has also allowed us to examine the stereoselectivity of the cyclization process for 8,5'-cycloadenosine formation and to follow 8-hydroxyadenosine formation and adenine release in irradiated poly A.

MATERIALS AND METHODS

Reagents

Authentic samples of (R)- and (S)-8,5'-cycloadenosine were synthesized by a published procedure involving the photolytic cyclization of the 2',3'-O-isopropylidene derivative of 5'-deoxy-5'-phenylthioadenosine (12). The final products of this procedure were identified by combined gas chromatography-mass spectrometry analysis (Dr. M. Dizdaroglu, personal communication) and were found to be identical to (R)- and (S)-8,5'-cycloadenosine obtained from the radiolysis of adenosine 5-monophosphate (5'-AMP) (5,7,13,14). Authentic 8-hydroxyadenosine was synthesized according to a published procedure (15) and characterized by infrared and ultraviolet spectroscopy and mass spectrometry. Alkaline phosphatase (Bovine Intestinal Mucosa, Type VII-S), nuclease P1 (NP1, Penicillium

citrinum), adenine, adenosine, 5'-AMP and polyadenylic acid (poly A, mol. wt. 140-500 kdal) were obtained from the Sigma Chemical Co. (St. Louis, MO) and used without further purification. Other chemicals were chromatographic or reagent grade and were obtained from local suppliers.

Irradiation

Irradiations were performed in a Gammacell 220 ^{60}Co gamma radiation source (Atomic Energy of Canada Ltd.) at a dose-rate of 58.7 Gy/min as measured by Fricke dosimetry [$G(Fe^{3+}) = 15.6$ (16)]. Solutions of poly A containing 697 µg/ml were made up in distilled water (pH 7.0) which had been purified and deionized before use by passage through a Barnstead three module NANOpure water purification system (supplied by Fisher Chemical Co.). The 2.0 ml samples of poly A were bubbled with nitrous oxide for 15 minutes prior to and then throughout the time of irradiation. The nitrous oxide was scrubbed free of oxygen by passage through a 0.1 $mol \cdot dm^{-3}$ sodium dithionite trap and freed of acidic impurities by passage through a 0.1 $mol \cdot dm^{-3}$ sodium carbonate trap. For comparison with the poly A results, 10^{-3} $mol \cdot dm^{-3}$ solutions of adenosine were irradiated to 1000 Gy at pH 7.0 under nitrous oxide as described for poly A.

Hydrolysis of Irradiated Poly A

The irradiated poly A was hydrolyzed with nuclease P1 following the procedure of Gehrke et al. (17). Briefly, 0.250 ml of a solution of nuclease P1 containing 7.5 units/ml enzyme in a buffer of 30

mmol·dm^{-3} sodium acetate (pH 5.3) and 2 mmol·dm^{-3} $ZnSO_4$ were added to 0.5 ml of poly A solution. The mixture was incubated for 18 h at 37° C.

The mixture of mononucleotide products was further hydrolyzed to the corresponding nucleosides with alkaline phosphatase. In a typical experiment 0.250 ml of a solution of alkaline phosphatase (25 units/ml of alkaline phosphatase in 0.1 mol·dm^{-3} sodium carbonate/bicarbonate buffer (pH 9.6) containing 1 mmol·dm^{-3} magnesium chloride) was added to the NP1 digest of the irradiated poly A and the mixture was incubated at 37°C for 18 h. This procedure produced an unchanging hydrolysate chromatogram free of detectable levels of products that could be detected in intentionally, partially hydrolyzed samples. In addition, the linear dose-yield response for adenosine destruction in the dose range 0-1000 Gy (ten 100 Gy increments; regression analysis; correlation coefficient 0.972) indicates that large doses of radiation to poly A do not detectably alter its hydrolysis by nuclease P1.

High-Performance Liquid Chromatography

High-performance liquid chromatography was performed on a Spectra Physics system incorporating a 8100 liquid chromatograph, a 8110 automatic sample injector, a 8440 variable wavelength detector set at 266 nm, and a 4270 computing integrator. A reversed-phase Supelco LC-18-S (4.6 mm x 25 cm) column which has been developed specifically for nucleoside chromatography was used with an eluent of 7.5 percent methanol in 0.02 mol·dm^{-3} ammonium formate (pH 4.0) at a flow rate

of 1.0 ml/min. The Supelco LC-18-S column gave a slightly different chromatogram from that observed in our previous study (18). Adenine release from irradiated poly A was measured prior to enzyme hydrolysis. The nucleoside analogs formed in irradiated poly A were chromatographed and quantitated following enzyme hydrolysis with nuclease P1 and alkaline phosphatase as described above.

ELISA Methodology

A detailed description of the ELISA technique has been reported previously and was followed without modifications (11).

RESULTS

Radiolytic adenine release from poly A was quantitated by means of HPLC prior to enzymatic digestion to limit the possibility of cochromatography with products resulting from enzyme hydrolysis. Enzymatic hydrolysis of poly A (irradiated in the presence of N_2O) with nuclease P1 and alkaline phosphatase resulted in the liberation of a number of modified products which were separated from unchanged adenosine using reversed-phase chromatography (Figure 1). Adenine, adenosine, (R)- and (S)-8,5'-cycloadenosine and 8-hydroxyadenosine were identified as radiolysis products by cochromatography with authentic samples. Other, as-yet-unidentified products were also observed. The yields of the identified radiolysis products as measured by HPLC, were quantified with reference to calibration curves using authentic samples. As reported elsewhere (18), the yield of the (R)-epimer in irradiated poly A predominates over that

of the (S)-epimer (Figure 2). Also included in Figure 2 is a measure of the total yield of 8,5'-cyclonucleoside. The yield of neither 8,5'-cyclonucleoside (Figure 2) nor 8-hydroxyadenosine (Figure 3) moieties was linear as a function of the dose between 0 and 1000 Gy for irradiated solutions of poly A. Adenine release from irradiated poly A, however, was linear with dose in this range, exhibiting a G value of 0.28 (Figure 3).

The G values for product formation in N_2O-saturated solutions of adenosine, 5'-AMP (5) and poly A irradiated at pH 7.0 in distilled water are listed in Table 1. Radiolytic adenine release is greater for adenosine and 5'-AMP (G = 0.60, 0.50, respectively) than for poly A (G = 0.28). The yield of the 8-hydroxyadenosine moiety is similar for the three compounds (G = 0.12 - 0.15). The yield of the 8,5'-cyclonucleotide is higher in 5'-AMP (G = 0.52) than is the corresponding cyclonucleoside in adenosine (G = 0.14) and poly A (G = 0.23). The ratio of damage at the C(5') as compared to the C(8) position, as represented by 8,5'-cycloadenosine formation and 8-hydroxyadenosine formation, respectively, may serve as an indication of damage distribution in irradiated adenine-based compounds. In this context, 5'-AMP had twice the yield of damage at the C(5') position compared to poly A and four times the yield of damage at this site compared to adenosine.

The ratio of the yields for (R)- and (S)-epimers of 8,5'-cycloadenosine is low for 5'-AMP (0.37) as compared to either adenosine (1.80) or poly A (1.56). The formation of the (R)-epimer,

therefore, is favored in irradiated solutions of adenosine or poly A, but is four to five times less favorable in irradiated 5'-AMP solutions at neutral pH.

The yield of 8,5'-cycloadenosine measured by a competitive ELISA in irradiated poly A prior to hydrolysis (see Ref. 11 for details), was compared with the total yield of the (R)- and (S)-epimers of 8,5'-cycloadenosine as measured by HPLC on the same sample. A good correlation between the two measurements is seen but the ELISA technique slightly overestimated the overall yield of 8,5'-cycloadenosine (Figure 4). It is conceivable that this difference is due to incomplete hydrolysis of irradiated poly A in the HPLC analysis. However, we have no chromatographic evidence for incomplete hydrolysis (see Materials and Methods). Furthermore, the levelling off in cyclonucleoside yield at higher doses which could indicate incomplete hydrolysis of poly A in the HPLC analysis is mirrored exactly by the ELISA assay where no hydrolysis is involved.

DISCUSSION

The advantages of ELISA techniques for measuring molecular lesions in irradiated nucleic acids include their potential for sensitive analysis. The immunochemical analysis removes the need for the hydrolysis of the irradiated nucleic acids to monomeric constituents prior to analysis and the sensitivity obviates the need for labelling the nucleic acids with radioactive markers prior

to irradiation as is required for some chromatographic (19,20) and chemical assays (21,22). The competitive ELISA technique (23), however, is an indirect assay and it is desirable where possible to have an independent check on the molecular specificity of the assay and its ability to provide quantitation of a particular molecular lesion in nucleic acids.

In the case of an immunochemical assay for thymine glycol, for example, it has been possible to test ELISA specificity and quantitation directly by virtue of the fact that thymine glycols in DNA can be quantified by an independent chemical assay (24,25). In the absence of such a direct assay, the approach taken here of characterizing an ELISA for a specific molecular lesion by chromatographic comparison in irradiated and gently hydrolyzed homopolymers of ribo- or deoxyribonucleotides may serve as an acceptable alternative. This might be particularly true in those cases where a knowledge of relative yields as distinct from absolute yields is sufficient. These could include studies of the action of repair enzymes on specific molecular lesions in irradiated nucleic acids or the effect of radiation modifiers on the yield of the molecular lesion. In such cases, it is only necessary to determine that the ELISA responds proportionally to changes in the amount of product present in the irradiated nucleic acid, as is clearly the case for the ELISA for 8,5'-cycloadenosine in irradiated poly A (Figure 4).

We had postulated that the levelling off in 8,5'-cycloadenosine yield at high dose might be due to hydrated electron destruction

of the cyclonucleoside (11,26) but could not rule out the possibility that the ELISA itself was sensitive to changing antibody affinity to the intact lesion as the polynucleotide substrate was destroyed nonspecifically at higher dose. The HPLC results now confirm that the ELISA, in fact, responds precisely to the actual yields of 8,5'-cycloadenosine. It seems reasonable to assume that the ELISA is responding in a similar fashion in irradiated DNA (11), the ultimate target of interest, where we do not at present have an independent quantitative assay for 8,5'-cyclodeoxyadenosine formation.

In addition to providing an independent calibration for the ELISA technique, the HPLC analysis reveals that the intramolecular cyclization proceeds stereoselectively to produce, predominantly, the (R)-epimer in irradiated poly A (Figure 2). As discussed in greater detail elsewhere (18), the adenosine moieties in an extended chain of poly A can take up a conformation about the C(4')-C(5') bond (gauche-trans, Figure 5, I) (27) which would tend to favor the formation of the (R)-epimer of 8,5'-cycloadenosine (Figure 5, III). The fact that the (R)-epimer does predominate (Figure 2) indicates that the intermediate reactive species involved in the cyclization (presumably the C(5') free radical, Figure 5, II) does not alter the ground state conformation significantly in poly A, nor, in all likelihood, in DNA.

With reference to the formation of the intramolecular crosslink between the C(8) and C(5') carbons in general, the anti-conformation with respect to the orientation of the adenine base about C(1')

is favored in purine nucleotides (28,29), putting C(8) of the base in close proximity to C(5'). From this point of view, it is not surprising that the intramolecular cyclization process is reasonably efficient in poly A (G = 0.23). In fact, cyclonucleoside formation rivals the rather more complex events at the sugar phosphate backbone which lead to adenine release (G = 0.28) (3). Adenine release, however, is distinguished from 8,5'-cyclonucleoside and 8-hydroxynucleoside formation in irradiated poly A in that its yield is linear over the dose range studied (Figure 3). This result is consistent with the suggestion (11) that the levelling off in 8,5'-cycloadenosine yield and, possibly, 8-hydroxyadenosine yield as well may be due to secondary radical reactions at these lesions.

The G value for 8-hydroxyadenosine formation in irradiated solutions of poly A (G = 0.12) was similar to that of the monomers (G = 0.14 - 0.15). On this basis it would appear that $^{\cdot}OH$ attack at the C(8) position of the adenine base is not hindered in the ribopolymer.

The ratio of the damage occurring at the C(5') position compared to that at the C(8) position, as reflected by 8,5'-cyclonucleotide(side) and 8-hydroxynucleotide(side) formation, respectively, is directly correlated with the number of ionizable groups at the C(5') carbon. This ratio is 0.93, 1.92 and 3.71 for adenosine, poly A and 5'-AMP, respectively. At neutral pH adenosine, poly A and 5'-AMP have 0, 1 and 2 negatively-charged groups, respectively, at the C(5') carbon. This correlation between the

number of ionizable groups attached to the C(5') carbon and the ability to form the C(8) - C(5') intramolecular bond provides additional support in favor of the hypothesis that an ionizable group adjacent to a carbon atom, such as the phosphate group in 5'-AMP or poly A, is capable of activating the carbon (i.e., C(5')) with respect to hydrogen abstraction resulting from hydroxyl radical attack (5,7). It is for this reason, and the electron spin resonance data which demonstrates the existence of C(5') radicals in irradiated nucleosides and nucleotides (30-34), that we believe that not only the C(4') (3) but also the C(5') position is an important point of $^{\cdot}OH$ attack in phosphorylated nucleotides.

In summary, the HPLC analysis of hydrolyzed, irradiated poly A has shown that the ELISA for 8,5'-cycloadenosine precisely reflects changes in the yield of this product in irradiated poly A. On this basis, the ELISA has considerable potential as a sensitive and specific assay for nucleic acid studies. Previous studies have shown that the ELISA can detect the cyclonucleoside at 10 Gy in poly A and 100 Gy in DNA (11). In an absolute sense, the HPLC calibration has shown that the ELISA slightly overestimates the actual yield of 8,5'-cycloadenosine in poly A. HPLC calibrations of the ELISA in homopolymers of ribonucleotides as described here for 8,5'-cycloadenosine in poly A might be useful in general although, at this stage, the results can be expected to provide approximate quantitation only for molecular lesions in more complex substrates such as DNA. The HPLC analysis has revealed that

(R)-8,5'-cycloadenosine is formed preferentially in irradiated poly A and may be the result of the conformation of the polymer chain. In monomers, the factors which govern the epimeric distribution of the C(8) - C(5') cyclization product appear to be different. Damage to the adenine base resulting in the formation of 8-hydroxyAMP, in the case of 5'-AMP, or 8-hydroxyadenosine in the cases of adenosine or poly A, irradiated under N_2O at neutral pH is quantitatively similar. Finally, the two events initiated by hydroxyl radical attack at the sugar phosphate backbone; that is, adenine release and 8,5'-cycloadenosine formation, show somewhat different dose yield responses.

TABLE I

G VALUES FOR PRODUCT FORMATION[1]

	Adenosine	5'-AMP[2]	Poly A[3]
Adenine	0.60	0.50	0.28
(R)-8,5'-cycloADO	0.09	0.14	0.14
(S)-8,5'-cycloADO	0.05	0.38	0.09
8-hydroxyADO	0.15	0.14	0.12
Σ Products	0.89	1.16	0.64
Ratio R/S	1.80	0.37	1.56
Ratio C(5')/C(8)	0.93	3.71	1.92

1) Irradiations performed under N_2O at pH 7.0.

2) Data from Ref. (5). In the case of irradiated 5'-AMP, the G value represents that of the corresponding nucleotides.

3) G value determined at 500 Gy.

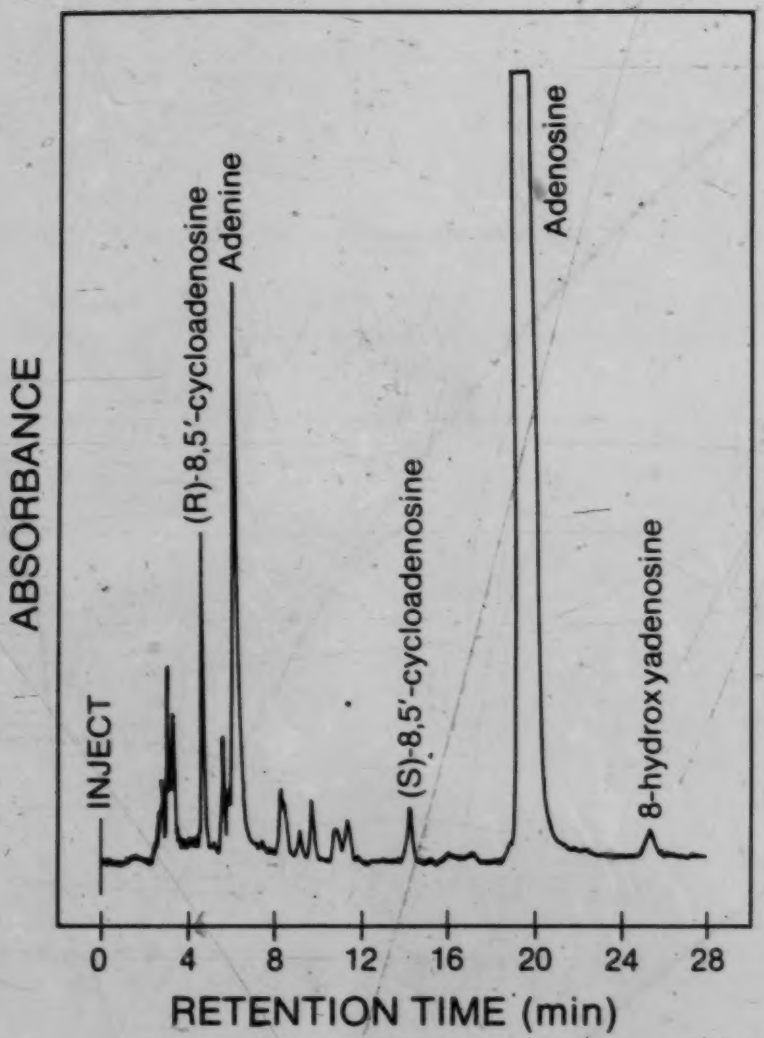

FIGURE 1: HPLC chromatogram of poly A irradiated to 200 Gy under N_2O at pH 7.0 and then hydrolyzed to the nucleoside level as described in the text. Unlabelled peaks represent unidentified radiolysis products.

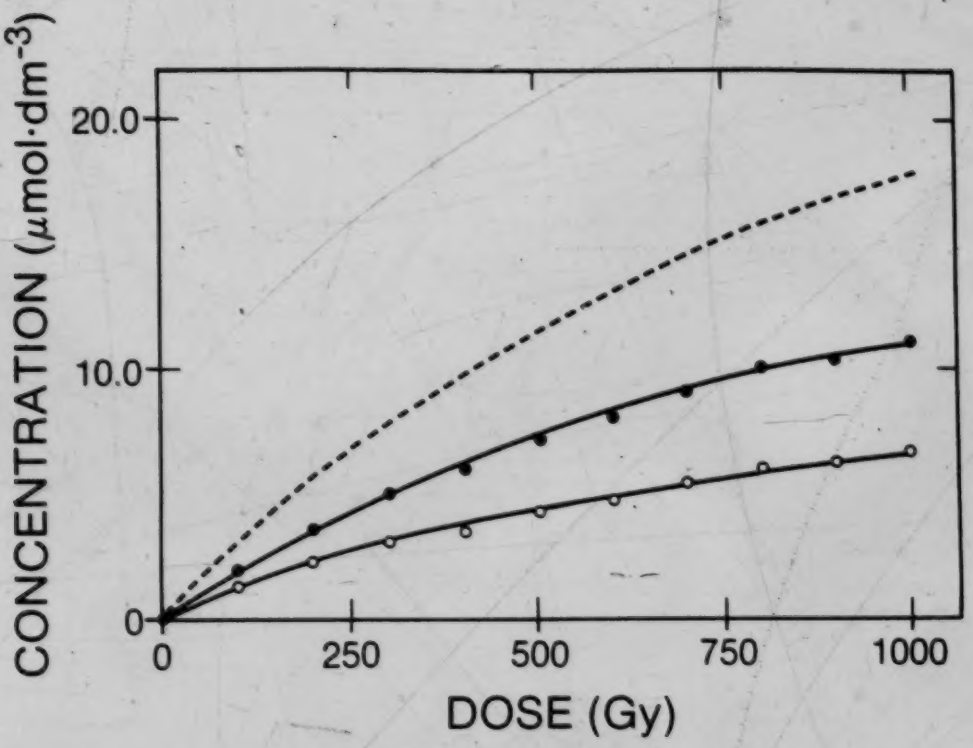

FIGURE 2: Yield of (R)-(●) and (S)-(○) 8,5'-cycloadenosine from irradiated poly A under N_2O at pH 7.0. Yields were measured by HPLC analysis and each point represents the average of three experiments. The SEM for each point was less than 4 percent. The dashed line represents the total yield of 8,5'-cycloadenosine obtained by the addition of the yield of each epimer at each dose.

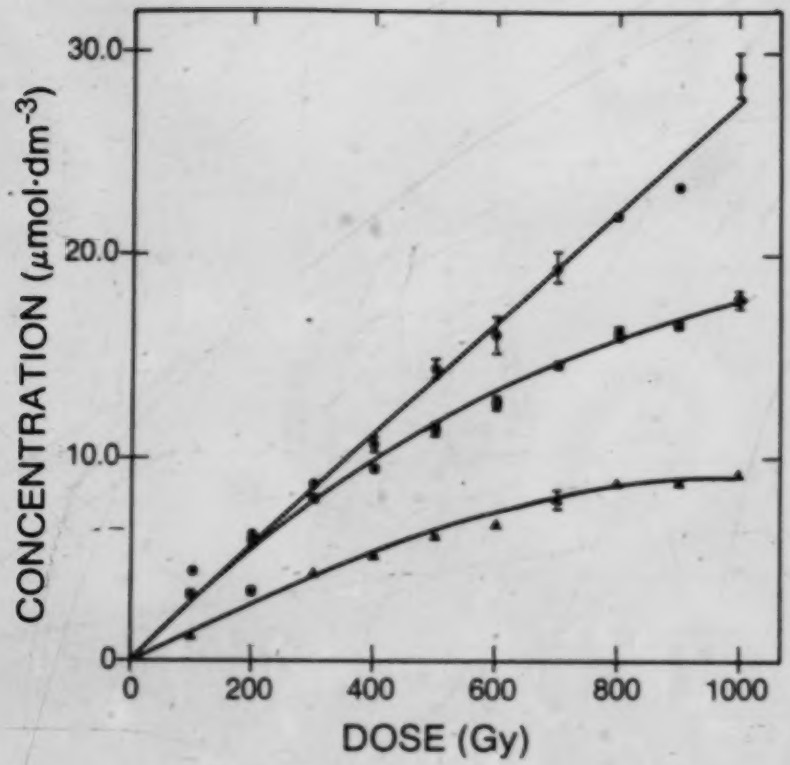

FIGURE 3: Comparison of the yields of 8,5'-cycloadenosine (■), 8-hydroxyadenosine (▲) and adenine release (●) as a function of dose for poly A irradiated under N_2O at pH 7.0. Each point represents the mean (± SEM) of three experiments. Note that the combined yield for 8,5'-cycloadenosine epimers is also illustrated in Figure 2.

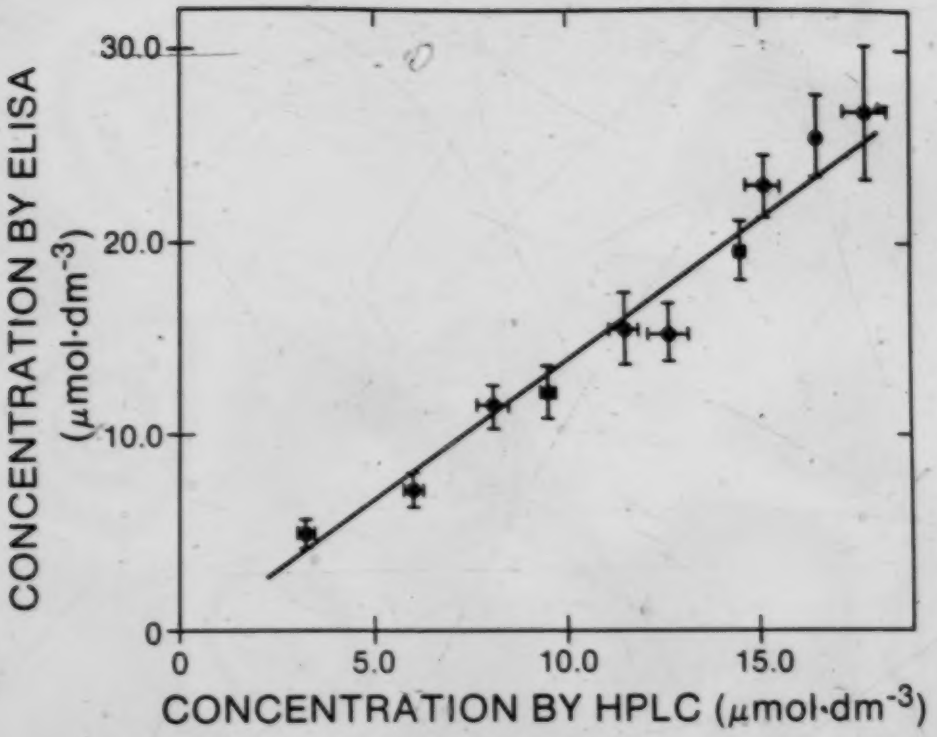

FIGURE 4: Comparison of the 8,5'-cycloadenosine yield in irradiated poly A solutions as measured by ELISA and HPLC assay respectively. Each point represents the mean (± SEM) of three experiments.

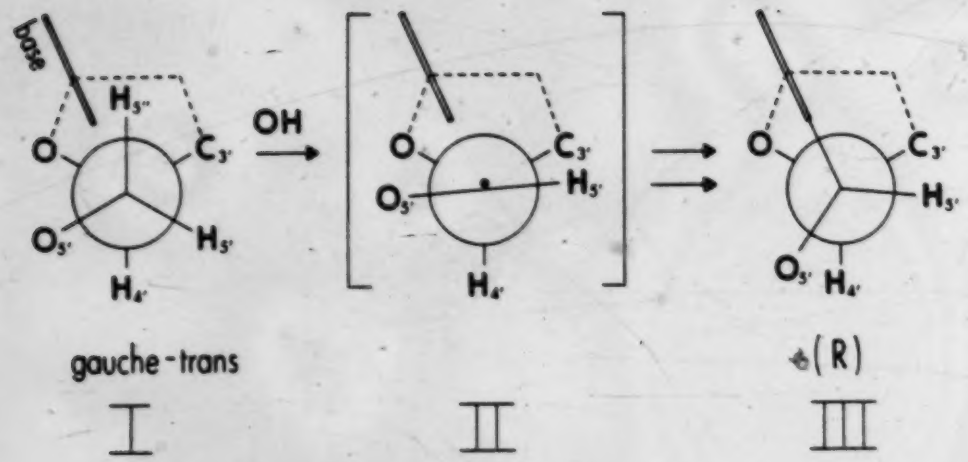

FIGURE 5: Newman projections showing the reaction sequence and conformation about the C(4')-C(5') bond which corresponds to adenosine (I); C(5') radical intermediate (II) and (R)-8,5'-cycloadenosine (III) following $^{\bullet}OH$ attack at the C(5') position.

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CHAPTER 8

STEREOCHEMICAL CONSIDERATIONS IN MOLECULAR RADIOBIOLOGY

Monitoring 8,5'-cyclonucleotide(side) formation in irradiated adenosine, adenosine 5'-monophosphate (5'-AMP) and polyadenylic acid (poly A) has brought to light the ability of certain functions within these compounds to direct the stereochemistry of radical reactions. Based on an examination of the relative yield of (R)- and (S)-epimers of 8,5'-cyclonucleotides(sides) it is postulated that in monomers intramolecular hydrogen bonding is an important factor governing stereoselectivity. However, in the case of poly A and presumably DNA, I propose that the importance of intramolecular hydrogen bonding is negligible while conformational factors, such as base stacking and base pairing interactions, direct stereoselectivity. This hypothesis is supported by experimental evidence presented in this thesis.

Intramolecular hydrogen bonding may play a predominant role in influencing the stereochemistry of 8,5'-cyclonucleotide(side) formation in irradiated adenine-containing monomers. In the case of 5'-AMP, the yield of the (R)-epimer of 8,5'-cycloAMP predominated at low pH (1). It was postulated that intramolecular hydrogen bonding between the ring oxygen and the monoprotonated 5'-phosphate group may favour a gauche-trans configuration about the exocyclic C(4')-C(5') bond (2) thus leading to predominance of (R)-8,5'-cycloAMP (1).

Deprotonation of the phosphate group (pK_a 6.6), which would remove the possibility of hydrogen bonding between the phosphate group and ring oxygen, apparently favours the trans-gauche configuration and the formation of the (S)-epimer of 8,5'-cycloAMP (1). Radiolysis of adenosine at neutral pH, on the other hand, favours the formation of the (R)-epimer of 8,5'-cycloadenosine (3). An examination of molecular models shows, in this case, that the gauche-trans C(4')-C(5') configuration could be stabilized by the formation of a hydrogen bond between the H-atom of the C(5') hydroxyl group and the ring oxygen leading to a predominance of the (R)-epimer of 8,5'-cycloadenosine (Figure 1). Indeed, formation of the (R)-epimer

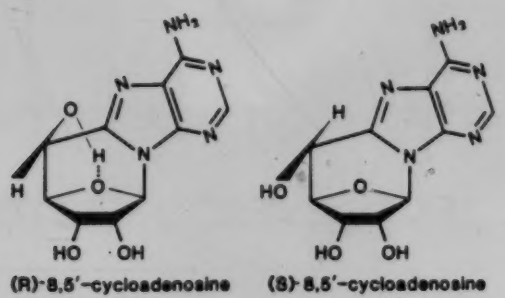

Figure 1: Molecular model illustrating the possibility of intramolecular hyrogen bonding for (R)-8,5'-cycloadenosine. Similar hydrogen bonding may also exist within the corresponding nucleotide. Intramolecular hydrogen bonding is not possible for the corresponding (S)-epimer.

of 8,5'-cycloadenosine is favoured in comparison to the (S)-epimer over the pH range 2 - 11 in irradiated solutions of adenosine (Fuciarelli, unpublished observations).

In the case of irradiated polymers, such as poly A or DNA, intramolecular hydrogen bonding may not be an important factor governing stereoselectivity. In fact, examination of molecular models reveals that the gauche-trans conformation about the C(4')-C(5') bond in the adenosine or deoxyadenosine moieties of nucleic acids predominates in extended polynucleotide chains which is compatible with intramolecular cyclization leading to the (R)-epimer (3,4). Stabilization of the gauche-trans conformation about the C(4')-C(5') bond may be attributed to a combination of hydrophobic forces driving the bases internally in relationship to the phosphodiester backbone, intrastrand "stacking" forces, and interstrand hydrogen bonding among the bases (5). The fact that the (R)-epimer does predominate indicates that formation of the intermediate C(5')-centered radical does not greatly change this conformation even in single-stranded poly A where there is the possibility of chain bending about the phosphate ester to produce the trans-gauche conformation which would lead to the (S)-epimer (3,4). Base stacking interactions in aqueous solutions of poly A buffered to neutrality may provide sufficient stability to prevent bending of the phosphate ester. In aqueous solutions of native DNA, chain bending would be much less likely to occur due to interchain hydrogen bonding and the tendency for the hydrophobic bases to project internally in relation to the

phosphodiester backbone. Indeed, Dickerson et al. (6) has reviewed evidence suggesting that because of the degree of stabilization in native DNA the entire chain represented by P-O(5')-C(5')-C(4')-C(3')-O(3')-P rotates as a semi-rigid unit and the overall P-P vector distance does not change. Therefore, the (R)-epimer of the 8,5'-cyclodeoxyadenosine moiety is expected to be even more predominant in native DNA than in an extended polymer consisting of only a single chain (denatured DNA or poly A) due to stabilization of the gauche-trans conformation. Having stated this, the relative proportion of (R)- and (S)-8,5'-cyclodeoxyadenosine has yet to be measured in irradiated solutions of native DNA.

In summary, stereoselectivity in 8,5'-cyclonucleotide(side) formation may be governed by intramolecular hydrogen bonding in monomers and conformational stabilizing factors in polymers. These conformational stabilizing factors might include base stacking in single-stranded extended polymers and a combination of base stacking, hydrophobic internalization and intermolecular hydrogen bonding amongst the bases in double-stranded polymers.

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CHAPTER 9

INTERACTION OF NITROAROMATIC RADIOSENSITIZERS WITH IRRADIATED POLYADENYLIC ACID AS MEASURED BY AN INDIRECT IMMUNOCHEMICAL ASSAY WITH SPECIFICITY FOR THE 8,5'-CYCLOADENOSINE MOIETY

INTRODUCTION

The end points of strand breakage and base release are the result of complex radical processes in the sugar phosphate backbone of irradiated nucleic acids (1). As a consequence, it is often difficult to interpret the outcome of the chemical modification of radiation response in terms of nucleic acid radicals when these endpoints are used. For example, the results of the interaction of nitroaromatic radiosensitizers with sugar phosphate radicals as measured by the strand break process depends on whether the 3' or 5' end of the internucleotide bond is involved and whether a pyrimidine or purine base is attached to the nucleotide (2-4). This complicates attempts to establish the role of nucleic acid radicals in the mechanism of radiosensitized cell lethality as embodied in the competition model of cell killing (5-10).

1. A version of this chapter has been accepted for publication. Fuciarelli, A.F., Mele, F.G., and Raleigh, J.A. Interaction of nitroaromatic radiosensitizers with irradiated polyadenylic acid as measured by an indirect immunochemical assay with specificity for the 8,5'-cycloadenosine moiety. Int. J. Radiat. Biol. (1987).

One type of radical event that leaves an unequivocal record of its occurrence in the sugar-phosphate backbone is C(5') hydrogen abstraction followed by intramolecular addition of the resulting C(5') radical to the C(8) position of a purine base to form a 8,5'-cyclonucleoside (IV, Scheme I) (11-17). Cyclonucleoside formation has recently been shown to occur in irradiated nucleic acids (15-20) and, in the case of polyadenylic acid (poly A), a G value of 0.23 for 8,5'-cyclonucleoside formation has been estimated by chromatographic means (16). It was of interest to now investigate the interaction of nitroaromatic radiosensitizers with the radical events which lead to this type of product.

The possibility that 8,5'-cyclonucleoside formation could be a useful probe for oxidative damage at the sugar-phosphate moiety, complementary to existing probes of base damage, has led to the development of a sensitive, immunochemical assay for 8,5'-cycloadenosine and its deoxy analog in nucleic acids (18). The immunochemical assay is used here to investigate the interaction of nitroaromatic radiosensitizers with the radical events leading to cyclonucleoside formation in irradiated polyadenylic acid (poly A). In analogy with oxygen (17,18) and in keeping with expectations from earlier studies of simple phosphate esters (2,21), cyclonucleoside formation has been found to be inhibited in a way which generally increases with increasing nitroaromatic electron affinity.

MATERIALS AND METHODS

Polyadenylic acid (poly A; mol. wt. 140-500 kdal) was purchased from the Sigma Chemical Co. (St. Louis, MO) and used as received. The nitrobenzene radiosensitizers were obtained from the Aldrich Chemical Co. (Montreal, QB). Misonidazole [1-(2-hydroxy-3-methoxypropyl)-2-nitroimidazole] was obtained from the National Institutes of Health (U.S.A.) through the good offices of Dr. V. Narayanan. The polyclonal antiserum and the methods for the enzyme-linked immunosorbent assay (ELISA) have been described previously (18). The sensitivity of the ELISA technique has allowed us to follow cyclonucleoside formation at a radiation dose which does not completely destroy the nitroaromatics and at yields of cyclonucleoside which are presently beyond the detection limits of HPLC techniques.

Throughout this chapter, the molecular lesion of interest is referred to as a cyclonucleoside rather than a cyclonucleotide. The cyclonucleoside is the irreducible structure of interest but it should be noted that the competitive ELISA used to measure cyclonucleoside content in poly A (Figures 1 and 3) requires the nucleotide, 8,5'-cycloadenosine 5'-monophosphate, as the competitive inhibitor of antibody binding to irradiated poly A.

Irradiations were performed in a Gammacell 220 ^{60}Co gamma radiation source (Atomic Energy of Canada Ltd.) at a dose-rate of 66.3 Gy/min as measured by Fricke dosimetry [$G(Fe^{3+}) = 15.6$ (22)]. Inhibition of 8,5'-cyclonucleoside formation in irradiated solutions

of poly A (365 μg/ml) in the presence of misonidazole was assayed by ELISA following irradiation to 100 Gy under either nitrogen or nitrous oxide. The solutions were prepared containing 0.1 mol·dm^{-3} phosphate buffer (pH 7.0) which was prepared with distilled water which had been further purified and deionized by passage through a Barnstead, three module NANOpure water purification system. The solutions were bubbled with the gases fifteen minutes before, and then throughout, the time of irradiation. Before passing into the solutions to be irradiated, the gases were freed of oxygen and acidic impurities (in the case of nitrous oxide) by sequential passage through gas wash bottles containing 0.1 mol·dm^{-3} sodium dithionite and 0.1 mol·dm^{-3} sodium carbonate, respectively.

The radiolytic destruction of the nitroaromatic compounds was measured by high-performance liquid chromatography (HPLC) in solutions containing poly A (355 μg/ml in 0.1 mol·dm^{-3} phosphate buffer, pH 7.0) and nitroaromatics (50 μmol·dm^{-3}), which were saturated with nitrous oxide and irradiated (0 - 250 Gy). The HPLC system included a Spectra Physics 8100 liquid chromatograph equipped with a Spectra Physics 8110 automatic sample injector, a Spectra Physics 8440 variable wavelength detector, and a Spectra Physics 4270 computing integrator. For analysis of compounds **11**, **12** and **13** (Table 1), a Waters μBondapak C_{18} column (3.9 mm x 30 cm) was used with an eluent of 50 per cent aqueous methanol containing 1 per cent acetic acid flowing at 1.0 ml per minute. The other substituted nitrobenzenes were separated on a Whatman Partisil 10 ODS column

(4.6 mm x 25 cm) with an eluent of 25 per cent aqueous acetonitrile at a flow rate of 1.0 ml per minute.

In order to determine the reactivity of the C(5') intermediate radical with the nitroaromatics, standard competition kinetic methods were used wherein the yield of 8,5'-cycloadenosine was determined at different ratios of poly A to nitroaromatic concentrations. Solutions of poly A (350 μg/ml in 0.1 $mol \cdot dm^{-3}$ phosphate buffer, pH 7.0) containing 10 - 500 $\mu mol \cdot dm^{-3}$ nitroaromatic were saturated with nitrous oxide and irradiated to 100 Gy. Formation of the 8,5'-cycloadenosine moiety was assayed using ELISA techniques.

RESULTS

The mechanism of formation of 8,5'-cyclonucleosides involves hydroxyl radical-induced hydrogen abstraction from C(5') followed by an intramolecular addition reaction to the C(8) position of the purine base in deoxygenated solution. The presence of the nitroaromatic radiosensitizer, misonidazole, leads to a decrease in the yield of 8,5'-cycloadenosine in irradiated poly A with a first order dependence on misonidazole concentration (Figure 1). The course of inhibition is similar in nitrogen- and nitrous oxide-saturated solution and roughly parallels that seen for dimethyl sulfoxide (dashed line, Figure 1) (18). The inhibition by misonidazole could be due to hydroxyl radical scavenging [$k_{OH + miso} = 7.1 \times 10^9$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$ (23)], to the interaction of misonidazole with the C(5') radical in poly A or, to a combination

of both.

The anticipated differential in $^{\cdot}OH$ reactivity among the nitrobenzenes (24) necessitated a measurement of the degree of destruction for each of the nitroaromatics in the irradiated solutions. This was achieved by HPLC analysis of nitroaromatic loss from aqueous solutions of poly A containing the substituted nitrobenzenes (50 $\mu mol \cdot dm^{-3}$) irradiated from 0-250 Gy (Figure 2). The least electron affinic nitroaromatics, p-nitrotoluene (1) and nitrobenzene (2), were destroyed to the greatest extent. It was determined in control experiments that neither of these nitroaromatics was lost by evaporation during the period of irradiation. With two or more strongly electron-withdrawing substituents on the benzene ring, the extent of destruction was largely independent of increasing electron affinity.

The reactivity of the C(5') radical in the competing processes of intramolecular addition leading to 8,5'-cyclonucleoside formation (k_2) and oxidation of this radical by the nitroaromatics (k_4) was evaluated by assaying 8,5'-cyclonucleoside formation in solutions of poly A irradiated to 100 Gy in the presence and absence of known concentrations of nitroaromatics (10 - 500 $\mu mol \cdot dm^{-3}$). To simplify the kinetic analysis, the contribution of hydroxyl radical scavenging by the nitroaromatics was not considered. In the competition for the C(5') radical intermediate, the extent of each reaction was proportional to the individual rate constants in solution. A relationship which can be used to describe the dependence of

8,5'-cyclonucleoside formation at various concentration ratios of poly A to nitroaromatic concentrations is the standard competition kinetic analysis:

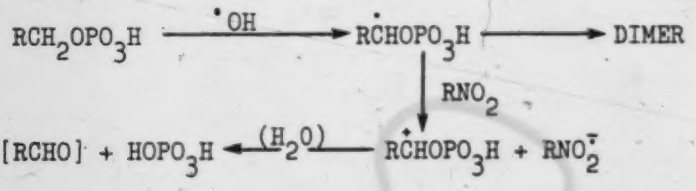
$$\frac{[\text{Product}]}{[\text{Product}]_{NA}} = 1 + \frac{k_4\,[NA]}{k_2\,[\text{poly A}]} \qquad (1)$$

where [Product] and $[\text{Product}]_{NA}$ represent the concentration of 8,5'-cyclonucleoside in the absence and presence of nitroaromatics (NA), respectively. Although reaction 2 is a unimolecular intramolecular cyclization, the k_2 should be independent of poly A concentrations, equation 1 can be used to estimate the relative reactivity of the C(5') radical towards the nitroaromatics by comparing the slopes of lines calculated from regression analysis of data illustrated in Figure 3. For the purposes of this analysis, the poly A concentration has been expressed in terms of equivalent mononucleotide concentration. The slopes, which represent the observed k_4/k_2 values for each nitroaromatic, are presented in Table 1 for comparison. The inhibition of cyclonucleoside formation by the nitroaromatics at a fixed concentration generally increased with increasing electron affinity (Table 1). Electron affinity is expressed in terms of the sum of Hammett σ constants ($\Sigma\sigma_x$) which have been shown to possess an additive linear free energy relationship with the redox potential of substituted aromatics (25). The reactivity of negatively-charged substituted nitrobenzenes [m-nitrobenzoic

acid (11), 3,5-dinitrobenzoic acid (12) and 2,4-dinitrobenzenesulfonic acid (13)] with the C(5') radical is much lower than uncharged nitroaromatics of similar electron affinity. By comparison, the methyl ester of compound 12, methyl 3,5-dinitrobenzoate (9), is uncharged under the experimental conditions and has the reactivity expected on the basis of its electron affinity. Two compounds, m-nitroacetophenone (4) and 3,5-dinitrobenzamide (8), have reactivities that exceed the reactivities of nitroaromatics with similar electron affinities.

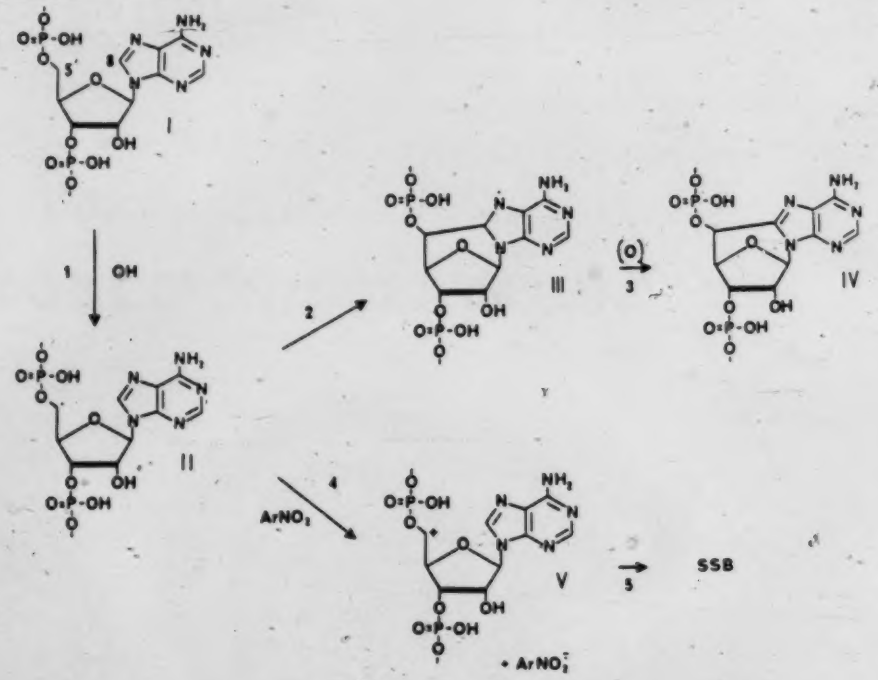
DISCUSSION

In the case of a simple phosphate ester such as O-phosphorylethanolamine, phosphate ester bond breakage is enhanced by nitroaromatic sensitizers in a way which increases systematically with sensitizer electron affinity (21). This was interpreted as being due to the one electron oxidation of an intermediate carbon radical which otherwise could dimerize in a cross-linking reaction in hypoxic solution (3,26). The one electron oxidation process is analogous to that occurring in pulse radiolysis studies of one electron oxidation of alcohol radicals by nitroaromatics (27).



While the enhancement of phosphate ester cleavage in simple model compounds is consistent with the proposed mechanism, the interaction of nitroaromatic radiosensitizers with the 3'- and 5'- phosphate ester bonds in nucleotides is far more complex (3,4) with the result that the strand break process is equivocal as a diagnostic tool for unravelling underlying radical events in the sugar phosphate moiety in nucleotides.

In contrast to the relatively complex processes leading to strand breakage, the initial radical events leading to 8,5'-cyclonucleoside formation in irradiated nucleotides are reasonably well-understood (Scheme I).



Scheme 1: Proposed mechanism for the radiosensitizer inhibition of 8,5'-cyclonucleoside formation in irradiated poly A (SSB = single strand break).

The process is initiated by hydroxyl radical attack at C(5') of the ribose ring followed by addition of the C(5') radical to the C(8) position of the purine base (11). Electron spin resonance studies have provided evidence for the existence of C(5') radicals in irradiated nucleotides and nucleosides (28-32) and the nucleophilic addition of alkyl radicals to C(8) of purine bases is a well-known phenomenon (33). The radical events subsequent to C(5') radical addition to C(8) are less well-understood. For mononucleotides, it is postulated that the intermediate radical disproportionates to produce the cyclonucleotide (11). The close approach of two intermediate radicals to accommodate disproportionation in a nucleic acid such as poly A, however, seems much less likely.

Nitroaromatic radiosensitizers can, in principle, interact with the radicals leading to cyclonucleoside formation in a number of ways. Hydroxyl radical scavenging by the nitroaromatics could inhibit cyclonucleotide formation by preventing C(5') radical formation so that it is not possible with a single radiosensitizer such as misonidazole to rule out $^{\cdot}OH$ scavenging as the basis of the inhibition of 8,5'-cyclonucleoside formation. The rate of hydroxyl radical attack on substituted benzene compounds to form $^{\cdot}OH$ adducts, however, has been shown to decrease with increasing aromatic electron affinity (24). This is opposite to the observed inhibition of cyclonucleoside formation which generally increases with increasing electron affinity (Table 1, Figure 3). The inhibition is more consistent with nitroaromatic oxidation of the intermediate C(5')

radical in competition with addition of the C(5') radical to the C(8) position of adenine (reaction 4, Scheme I). (A closely related and presently indistinguishable alternative involving addition of the nitroaromatic to the C(5') radical cannot be ruled out (34). Similarly nitroaromatic oxidation of, or addition to, the N(7)-centered radical or to the 8,5'-cycloadenosine moiety cannot be ruled out.) Further support for this mechanism, which was proposed previously as a possible mechanism of sensitized strand breakage (21), comes from an examination of the radiolytic destruction of the nitrobenzenes in the presence of poly A.

Nitroaromatic destruction in the nitrous oxide-saturated solutions is expected to be due primarily to $^{\cdot}OH$ attack since, on the basis of relative kinetic reactivities, hydrated electrons will be scavenged preferentially by N_2O ($[N_2O]$ = 16 $mmol \cdot dm^{-3}$, $k(e^-_{aq} + N_2O) = 5.6 \times 10^9$ $dm^3 \cdot mol^{-1} \cdot s^{-1}$ (35,36)). The extent of destruction among the more electron affinic nitrobenzenes (Compounds 3-13, Figure 2) is relatively small and constant over the electron affinity range where major changes in 8,5'-cycloadenosine formation are occurring (Figure 3) indicating that $^{\cdot}OH$ scavenging cannot be the primary source of this inhibition. In the cases of nitrobenzene and p-nitrotoluene substantial destruction and, presumably, $^{\cdot}OH$ scavenging is observed. Nevertheless, little or no inhibition of cyclonucleoside formation is seen. It might be possible that secondary, $^{\cdot}OH$ adduct radicals from $^{\cdot}OH$ attack on the nitrobenzenes are capable of initiating cyclonucleoside formation by abstracting hydrogen atoms

from the C(5') position of the ribose ring thereby offsetting the effect of ·OH scavenging. This process would be much less important for the most electron affinic substituted nitrobenzenes due to their diminished reactivity with hydroxyl radicals. An alternative possibility for the results with nitrobenzene and p-nitrotoluene arises from a consideration of the mechanism of 8,5'-cyclonucleoside formation (Scheme I). The final step in cyclonucleoside formation involves a one electron oxidation of the radical intermediate III. As noted above, in mononucleotides this is believed to be accomplished in a radical-radical disproportionation reaction. It is conceivable, however, that nitroaromatic compounds might act as oxidants in this step in which case the yield of 8,5'-cyclonucleotide might be enhanced (cf. 37). Such is clearly not the case for the most electron affinic, uncharged nitrobenzenes. If indeed they are capable of oxidizing radical III which would lead to an increase in the yield of 8,5'-cyclonucleosides, then the effect is completely offset by their scavenging of the C(5') radical (II). However this process could, in part, account for the apparent lack of effect of nitrobenzene and p-nitrotoluene if the threshold electron affinity for the one electron oxidation of III were lower than that for II. While the seemingly unexceptional effect of nitrobenzene and p-nitrotoluene shown in Figure 2 is possibly fortuitous due to the differing oxidation potentials of II and III, the trend linking decreased cyclonucleoside formation in irradiated poly A with increasing nitrobenzene electron affinity is established by a series of

nitrobenzenes (Table 1) whose extent of destruction and, presumably, reactivity with $^{\cdot}OH$ is relatively constant.

A number of nitroaromatics have been identified which clearly deviate from the general trend linking decreased cyclonucleoside formation with increasing nitrobenzene electron affinity. Compounds **11**, **12** [pK_a 3.5 and 2.7 respectively; (38)] and **13** [acidity comparable to perchloric acid; (39)] have a fixed negative charge under the conditions of the experiments. The relative reactivity of these compounds, especially **12** and **13**, is much lower than uncharged nitroaromatics with similar electron affinity (Table 1; Figure 3). Although thermodynamically favourable, the lack of inhibition of cyclonucleotide formation might be kinetically unfavourable due to charge repulsion of these substituted nitrobenzenes by the negatively-charged phosphate groups in poly A. This would prevent the close approach of the nitroaromatics to the C(5') radical on the phosphodiester backbone of irradiated poly A and thereby diminish the probability of nitroaromatic-induced oxidation of the C(5') radical. This hypothesis was supported in a comparison of the relative reactivity of 3,5-dinitrobenzoic acid (**12**; k_4/k_2 = 26) with that of the uncharged methyl ester (**9**; k_4/k_2 = 105). Three additional nitroaromatics deviated from the general relationship including m-nitrobenzonitrile (**6**) whose relative reactivity (k_4/k_2 = 27) falls below, and m-nitroacetophenone (**4**) and 3,5-dinitrobenzamide (**8**) whose relative reactivities exceed (k_4/k_2 = 109 and 212, respectively), that expected from a comparison of the relative rate

of reactivity of nitroaromatics with similar electron affinities. The reasons for these deviations are not clear.

In summary, it has been possible to demonstrate an interaction of nitroaromatic radiosensitizers with a specific radical event in irradiated poly A by means of an immunochemical assay for the cyclonucleoside molecular lesion. In general, the interaction is dependent on sensitizer electron affinity and is consistent with a one electron oxidation of the C(5') radical which is the precursor to cyclonucleoside formation. Negatively-charged nitroaromatics are much less effective in this interaction than uncharged nitroaromatics with equivalent electron affinity. The results indicate that the immunochemical assay for 8,5'-cyclonucleoside formation can serve as a useful probe for the interaction of chemical modifiers with a specific radical event in the sugar phosphate backbone of irradiated nucleic acids.

TABLE 1

RATE CONSTANTS FOR INTERACTION OF NITROAROMATICS WITH THE C(5') RADICAL INTERMEDIATE BASED UPON COMPETITION KINETICS USING SOLUTIONS OF POLYADENYLIC ACID (350 μg/ml, pH 7.0) IRRADIATED TO 100 Gy UNDER NITROUS OXIDE.

	NITROAROMATIC	ELECTRON AFFINITY[1] ($\Sigma \sigma_X$)	k_1/k_2[2]
UNCHARGED			
1.	p-NITROTOLUENE	-0.17	44
2.	NITROBENZENE	0.0	24
3.	m-NITROBENZAMIDE	+0.28	30
4.	m-NITROACETOPHENONE	+0.38	109
5.	p-NITROACETOPHENONE	+0.52	65
6.	m-NITROBENZONITRILE	+0.68	27
7.	m-DINITROBENZENE	+0.71	110
8.	3,5-DINITROBENZAMIDE	+0.99	212
9.	METHYL 3,5-DINITROBENZOATE	+1.02	105
10.	3,5-DINITROBENZONITRILE	+1.39	103
NEGATIVELY CHARGED			
11.	m-NITROBENZOIC ACID	+0.10	11
12.	3,5-DINITROBENZOIC ACID	+0.81	26
13.	2,4-DINITROBENZENESULFONIC ACID	+0.93	20

(1) The sum (Σ) of Hammett sigma constants (σ_X, 40) are calculated with respect to nitrobenbene (σ_X = o where X = H).

(2) Increasing values of k_1/k_2 indicate increasing inhibition of 8,5'-cyclonucleoside formation. Slopes obtained from lines fitted using least square linear regression analysis.

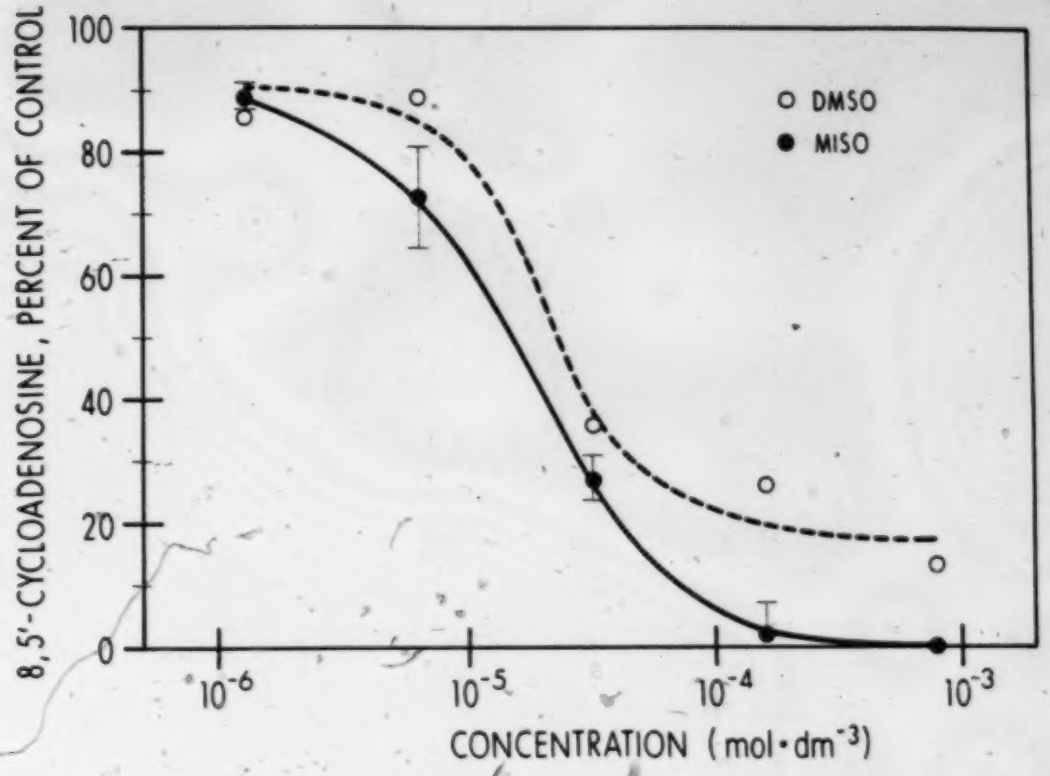


Figure 1: Inhibition of 8,5'-cyclonucleoside formation in irradiated poly A solutions (365 µg/ml) by misonidazole (●) and dimethylsulfoxide (DMSO) (○). The data points for misonidazole are the mean ± SEM for three experiments. The data points for DMSO are taken from Fuciarelli et al. (18). Solutions saturated with nitrous oxide and irradiated to 100 Gy at pH 7.0.

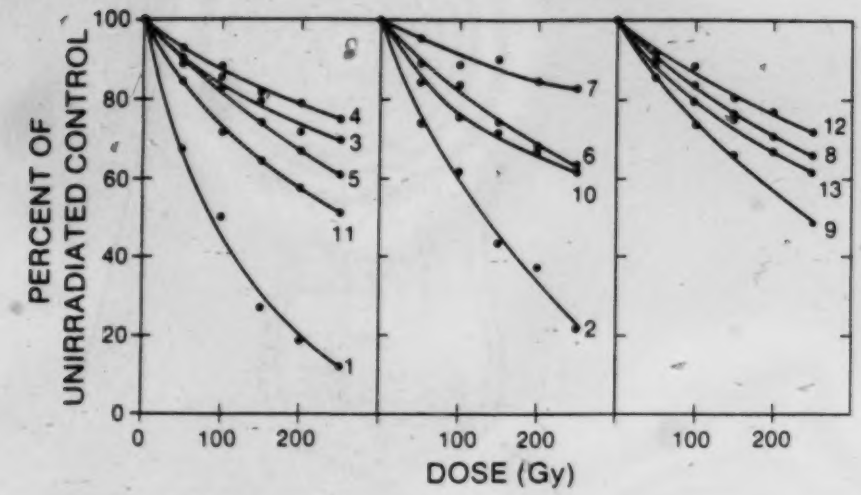


Figure 2: Radiolytic destruction of nitroaromatics (50 $\mu mol \cdot dm^{-3}$) in aqueous solutions of poly A (355 µg/ml) containing 0.1 $mmol \cdot dm^{-3}$ phosphate buffer (pH 7.0). Identities of the nitroaromatics are given in Table 1.

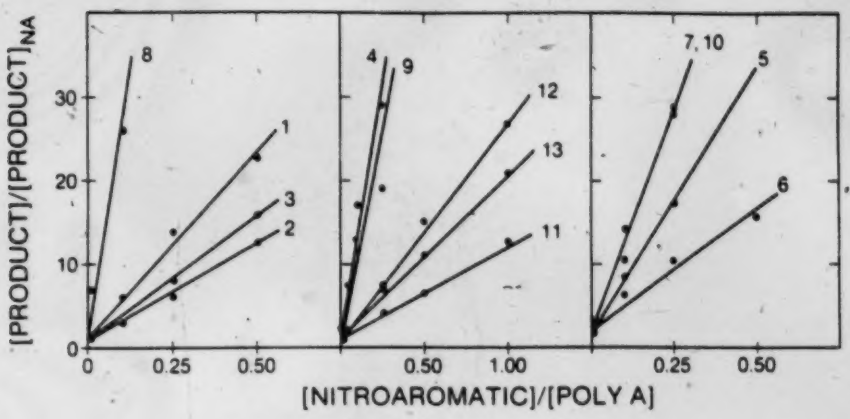


Figure 3: Relative reactivity of the C(5') radical intermediate formed from radiolysis of poly A (350 µg/ml; dose : 100 Gy) toward a series of substituted nitrobenzenes. Identity of the nitroaromatics is given in Table 1.

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CHAPTER 10

APPLICATION OF ULTRASENSITIVE ENZYME RADIOIMMUNOASSAY (USERIA) TO THE STUDY OF 8,5'-CYCLONUCLEOSIDE FORMATION IN IRRADIATED NUCLEIC ACIDS

INTRODUCTION

Ionizing radiation introduces a plethora of lesions into irradiated nucleic acids including strand breaks (single and double), crosslinks (DNA-DNA and DNA-protein) and specific molecular modifications to the sugar and base components (1-6). Characterization of these lesions is necessary to elucidate the mechanisms leading to carcinogenesis, mutagenesis, aging and cell lethality.

Characterization of specific molecular modifications to nucleic acid constituents is a difficult undertaking due to the requirement for analytical techniques that offer both high sensitivity and specificity. Techniques emerging from analytical chemistry, such as high-performance liquid chromatography (7-9), combined gas chromatography - mass spectrometry (10-14), and combined high-performance liquid chromatography - tandem mass spectrometry (15), have successfully met these criteria and have been applied in radiation chemical studies. Immunochemical techniques which use polyclonal antiserum or monoclonal antibodies specific to radiation-induced molecular products, have been developed to detect product formation in irradiated nucleic acids. In this context,

immunochemical assays have been developed to detect nucleic acid base damage as exemplified by thymine glycols (16,17), 5-hydroxymethyldeoxyuridine (18,19), 8-hydroxyadenine (20) and 8,5'-cyclodeoxyadenosine (21) in irradiated DNA. Application of immunochemical methodology to address questions in radiation chemistry has also been demonstrated (9,21,22). However, to date there has been no report in the literature characterizing any purine base damage in DNA extracted from irradiated cells.

In an effort to detect purine base damage in DNA extracted from irradiated cells, an ultrasensitive modification (23,24) has been applied to the basic enzyme-linked immunosorbent assay (ELISA) described by Fuciarelli et al. (21) for 8,5'-cyclodeoxyadenosine. The resultant assay, ultrasensitive enzyme radioimmunoassay (USERIA), was developed in an effort to increase the sensitivity of the immunochemical assay. The specificity remained unchanged relative to the basic ELISA since this parameter is governed by the nature of the antiserum. The sensitivity of the USERIA is compared with that of the ELISA. In addition, the methodology used in an attempt to measure 8,5'-cyclodeoxyadenosine formation in the DNA extracted from irradiated V79 cells is presented.

METHODS AND MATERIALS

1. Irradiation of Nucleic Acids in Solution

Samples of native calf thymus DNA (Sigma Chemical Co., St. Louis, MO) were prepared in 100 mmol·dm^{-3} phosphate buffer (pH 7.00)

at a concentration between 350 - 360 μg/ml. These samples were bubbled with nitrous oxide for 15 minutes prior to, and then throughout the time of irradiation. Nitrous oxide was scrubbed free of oxygen by passage through a 0.1 $mol \cdot dm^{-3}$ sodium dithionite trap and scrubbed free of acidic impurities by passage through a 0.1 $mol \cdot dm^{-3}$ sodium carbonate trap. Irradiation was performed in a Gammacell-220 ^{60}Co gamma radiation source (Atomic Energy of Canada Ltd.) at a dose rate of 28.1 Gy/min as determined by Fricke dosimetry [$G(Fe^{3+})$ = 15.6 (25)]. Each sample was diluted ten-fold in PBS-Tween (pH 7.4) (see (21) for composition of buffer) prior to product analysis to minimize nonspecific cross-reactivity. The DNA was assayed in the native form.

2. Immunochemical Assay

Antiserum with specificity to (R)-8,5'-cyclcAMP was prepared as described previously (21). The antiserum was used to develop an indirect, competitive enzyme-linked immunoscrbent assay (ELISA) to assay 8,5'-cyclonuclectide formation in irradiated nucleic acids. Competitive inhibition curves and the methodology have been reported previously (21). An ultrasensitive modification of the standard ELISA technique, USERIA reported in the literature (23,24) has increased the sensitivity of the standard ELISA technique. The modification involves the substitution of a radioactive substrate for p-nitrophenyl phosphate used in the standard ELISA. In the present study the hydrolysis of [^{3}H]-adenosine 5'-monophosphate (New England Nuclear, specific activity = 4.1×10^{11} Bq/mmol) to

[^{3}H]-adenosine, as a function of the amount of goat antirabbit IgG-alkaline phosphatase, was performed in 10% diethanolamine buffer (pH 9.6; see (21) for composition) for 18h at 37°C. Following incubation, 0.05 ml of solution was applied to a 0.2 ml suspension of AG1-X8 anion exchange resin (formate form; BioRad Laboratories Ltd., Richmond, CA). Greater than 90% of the [^{3}H]-adenosine was eluted following the addition of 0.050 - 0.350 ml of 10% diethanolamine (pH 9.6) whereas [^{3}H]-adenosine 5'-monophosphate, by virtue of its negatively-charged phosphate groups, remained bound to the column. The fractions containing [^{3}H]-adenosine were collected, 20.0 ml of scintillation fluid (Scinti-Verse-I, Fisher Scientific Co., Fairlawn, NJ) were added and the samples were counted in a liquid scintillation counter (Beckman LS 7000). Background [^{3}H]-adenosine activity resulting from nonspecific binding of antiserum to the microtiter wells was subtracted from the experimental samples.

3. Preparation of DNA Extracted from Irradiated Cells

3.1 Cell Line and Culture Conditions

Stock cultures of V79-WNRE Chinese hamster fibroblast cells were grown to confluency (30 x 10^6 cells) in 150 cm^3 tissue culture flasks (Corning Glass Works, Corning, NY) in Minimal Essential Medium [(MEM) cell nutritional components from Gibco Laboratories, Grand Island, NY, supplemented with penicillin and streptomycin] and fetal calf serum (12.5% v/v). The cell numbers for this experiment were measured by a Coulter Counter (Coulter Electronics Ltd., Halesh,

FL) following dilution in Isoton II (Coulter Electronics Ltd., Halesh, FL).

3.2 Depletion of Intracellular Thiols

Depletion of glutathione was accomplished by the addition of D,L-buthionine-S-R-sulfoximine (BSO; Chemical Dynamics Corporation, South Plainfield, NJ) to the confluent cell layer. Fifty milliliters of MEM containing 0.05 $mmol \cdot dm^{-3}$ BSO were added to three 150 cm^3 flasks containing a layer of confluent cells. Fifty milliliters of MEM were also added to three 150 cm^3 flasks containing a layer of confluent cells. The glutathione-depleted cells were allowed to incubate for 18 hours at 37°C followed by a rinse in Spinner Minimal Essential Medium (Spinner MEM, Gibco Laboratories, Grand Island, NY, containing 7% fetal calf serum). Non-protein sulfhydryls were also depleted from glutathione-depleted cells by the addition of 50 ml of Spinner MEM containing 0.1 $mmol \cdot dm^{-3}$ diethyl maleate (DEM; Sigma Chemical Co., St. Louis, MO) (26). The three non-depleted "control" flasks were rinsed and 50 ml of Spinner MEM was added prior to the one hour incubation at 37°C. Following DEM treatment, cells were kept cold to prevent recovery of the sulfhydryl level to that of untreated cells (26). Intracellular glutathione levels were not measured during this experiment but are reportedly reduced to less than 10% of controls by this procedure (26).

3.3 Degassing Cells

The formation of 8,5'-cyclonucleosides is inhibited at liquid-phase oxygen concentrations exceeding 1.6% pO_2 (Chapter 4).

With this in mind, it was necessary to use the techniques of Koch (27) to ensure that the oxygen concentration was reduced below this level. Briefly, the cells were rinsed, trypsinized, gently spun down (200 g for ten minutes in a Beckman Model TJ-6 centrifuge) to remove the trypsin-containing medium and the contents of each of three flasks were resuspended in approximately 10 ml of ice cold Spinner MEM medium. The result of this step is two, 10 ml suspensions of cells, one being depleted of glutathione and other nonprotein sulfhydryls ("thiol depleted"), the other being untreated in this respect. Chilled glass petri dishes [50 mm diameter; modified according to (27)] were inoculated with 35×10^6 cells (in approximately 1.0 ml of medium) of either thiol-depleted or untreated cell suspensions using an Eppendorf pipette. The dishes were stacked in aluminum chambers (27) in the following order (bottom to top): one empty "spacer" dish, one dish containing thiol-depleted cells, one dish containing untreated cells and the uppermost dish (uncovered without cells) containing 4.0 ml of a filtered solution of 0.1 $mol \cdot dm^{-3}$ sodium dithionite and 0.1 $mol \cdot dm^{-3}$ sodium carbonate. This latter solution is used to remove the last traces of oxygen from the chambers. The chambers were sealed and kept ice cold during the subsequent 30 minute degassing period. The chambers were connected to a manifold and the oxygen was removed by a series of eight gas changes involving the evacuation of the gas inside the chambers with a vacuum pump to about 70 mm Hg and the addition of nitrogen (< 5 p.p.m. O_2; Linde, Union Carbide, Edmonton, AB) to about 700 mm Hg. On the eighth gas change, the pressure was brought to 600 mm Hg to maintain vacuum within the chambers.

3.4 Irradiation of Cells

The chambers were irradiated in a Gammacell 220 ^{60}Co gamma radiation source (Atomic Energy of Canada Ltd.) at a dose-rate of 18.0 Gy/min to the following doses: 0, 10, 50, 100, 250, 500, 750 and 1000 Gy. The temperature of the cells was maintained at 0°C by packing the Gammacell chamber with ice. The absorbed dose was calculated by Fricke dosimetry (25).

3.5 Extraction of DNA From Cells

The irradiated cell suspensions were transferred to 1.0 ml centrifuge tubes after dislodging the attached cells with a rubber scraper. The cells were spun down (2000 g for 2.0 min) in a Fisher Model 59 centrifuge (Fisher Scientific Co., Edmonton, AB), rinsed with 1.0 ml of ice cold Spinner MEM medium and pelleted again. The cells were lysed by the addition of 0.2 ml of a solution of 0.1% sodium dodecyl sulphate (pH 7.2). Ribonucleic acids were digested by adding 0.25 units of Ribonuclease (Aspergillus clavatus, Sigma Chemical Co., St. Louis, MO) and incubating the solutions for 2 hours at 37°C. Proteinase K (0.02 mg in 0.01 ml of phosphate buffered saline, Tritirachium album; Type XI, Sigma Chemical Co., St. Louis, MO) was added to the solutions, which were left overnight at 37°C.

High molecular weight DNA was collected from each solution by size exclusion chromatography. Chromatographic columns were prepared from straight-walled 0.4 ml plastic centrifuge tubes (polyethylene Micro test tubes, BioRad Laboratories Ltd., Richmond, CA) from which the bottoms were cut off. A slug of glass wool was inserted into the open-ended tube to hold the resin and a suspension

of 0.2 ml of Biogel A-1.5 M (200 - 400 Mesh) (BioRad Laboratories Ltd., Richmond, CA) was carefully layered on top of this with a Pipettman (Gilson) pipette. The gel was washed with two, 0.2 ml washes of phosphate buffer (pH 7.2) before 0.1 ml of the lyzed cell solution was carefully layered on top. High molecular weight DNA was collected in 0.05 ml fractions and the chromatography was followed by the ethidium bromide fluorescence assay (28,29). Typically, high molecular weight DNA eluted after the addition of 0.10 - 0.45 ml of phosphate buffer (pH 7.2).

RESULTS AND DISCUSSION

Immunochemical techniques offer the potential of good sensitivity and specificity for product analysis. The specificity of an immunoassay is determined primarily by the inherent specificity of the antigen-antibody reaction and, to a lesser extent, by the efficiency of the technique used to separate reacted from unreacted products. The sensitivity of enzyme immunoassays is determined by the interaction of the antibodies with the antigen and by the kinetics of the enzyme-substrate reaction (23). The ultimate expression of the sensitivity of an enzyme immunoassay is the limit of detectability which is determined by the specific activity of the enzyme bound to the immunoreactant, detectability of the products of enzyme interaction, and an acceptable signal-to-noise ratio (23,30). Conventional ELISAs fail to attain extremely high sensitivities because the "noise" (nonspecific binding of the enzyme-labelled

antibody) is appreciably large in relation to the "signal" (specific antibody-antigen interactions). In addition, many enzyme immunoassays are based on the alkaline phosphatase p-nitrophenyl phosphate enzyme-substrate combination. The hydrolytic by-product of this enzyme reaction is inorganic phosphate which is known to cause a concentration-dependent inhibition of alkaline phosphatase activity resulting in nonlinear kinetics over a period of 2-3 hours. Finally, a high concentration of p-nitrophenyl phosphate is required in the conventional ELISA which, in combination with the plateauing of response resulting from enzyme inhibition due to the presence of inorganic phosphate in experimental samples, leads to a low signal-to-noise ratio.

Ultrasensitive modification to the ELISA involves replacing the conventional substrate, p-nitrophenyl phosphate, with a radioactively-labelled substrate which in the present study was [^{3}H]-adenosine 5'-monophosphate. Enzyme hydrolysis of this compound by alkaline phosphatase yields [^{3}H]-adenosine, which can easily be separated from the unreacted substrate. This modification primarily increases the signal-to-noise ratio to attain higher sensitivity. Linear enzyme-substrate kinetics are maintained in USERIA for greater periods of time (i.e. 24 hrs) as compared to ELISA ($\sim$2-3 hr) due to the 10^5-fold lower concentration of inorganic phosphate generated in the USERIA. In addition, radioactively-labelled compounds permit greater sensitivity due to lower detection limits as compared with spectrophotometric assay used in the ELISA. In combination, these

factors provide an amplification factor for the USERIA, permitting increased sensitivity as a result of increasing the signal-to-noise ratio.

Increased sensitivity for product detection using USERIA as compared to ELISA has been reported for detection of cholera toxin (31), rotovirus (31), acetylaminofluorene-DNA adducts (32), benzo(a)pyrene-DNA adducts (33,34) and aflotoxin B_1 - modified DNA (35,36). In the present study, USERIA was compared to ELISA to determine if the limit of detectability of 8,5'-cyclodeoxyadenosine in nitrous oxide-saturated solutions of native calf thymus DNA was lowered. Indeed, the limit of detection for 8,5'-cyclodeoxyadenosine was 10 Gy in USERIA, compared to a factor of 20 greater (200 Gy) for ELISA (Figure 1). Although this study was suitable for

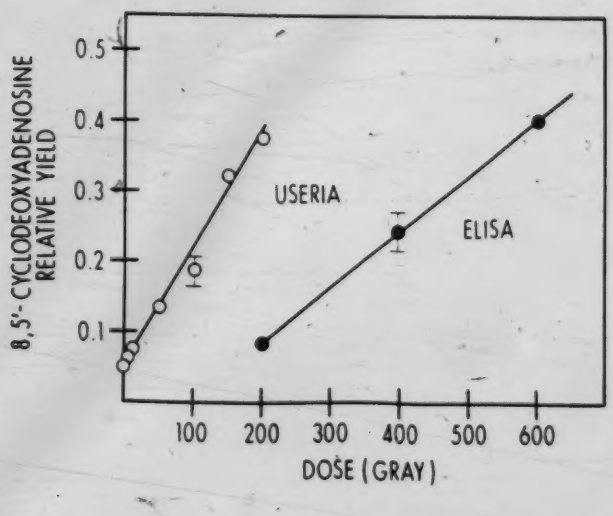

Figure 1: Comparison of the sensitivity of ELISA and USERIA for the measurement of 8,5'-cyclodeoxyadenosine in nitrous oxide-saturated aqueous solutions of calf thymus DNA (360 µg/ml phosphate buffer at pH 7.0).

establishing the respective limits of detectablity for USERIA and ELISA in terms of radiation dose, the slopes of the dose yield plots, and hence the G values for 8,5'-cyclodeoxyadenosine formation are different.

The ELISA technique has been calibrated with respect to HPLC (9) and may be used to estimate the expected yield of 8,5'-cyclodeoxyadenosine in DNA extracted from irradiated cells. The yield of 8,5'-cyclodeoxyadenosine in solutions of DNA irradiated to 1000 is 0.51 $\mu mol \cdot dm^{-3}$ on the basis of HPLC calibration of the ELISA assay. The efficiency of hydroxyl radical attack on DNA is decreased in the cellular environment as a result of the presence of scavenging species. By analogy with the strand-break process, the yield of DNA single-strand breaks (SSB) decreases by a factor of approximately 100 if one compares the yield of SSB from DNA irradiated in solution [0.2 - 0.3 $SSB \cdot krad^{-1} \cdot dal^{-1}$ (37)] and SSB fron DNA isolated from irradiated cells [0.0025 $SSB \cdot krad^{-1} \cdot dal^{-1}$ (38)]. Assuming that a similar decrease in yield would also occur for 8,5'-cyclodeoxyadenosine in DNA extracted from irradiated cells, the estimated yield of this product at 1000 Gy would be 0.005 $\mu mol \cdot dm^{-3}$. Since the assay is performed at a DNA concentration equivalent to 100 $\mu mol \cdot dm^{-3}$ in mononucleoside, this yield corresponds to one 8,5'-cyclodeoxyadenosine moiety per 2.0×10^4 unchanged mononucleoside residues.

The limit of detectability of the ELISA technique occurs at a dose of 200 Gy at which the uncorrected yield of 8,5'-cyclodeoxyadenosine is 0.09 $\mu mol \cdot dm^{-3}$ (Figure 1). Using the

calibration technique (9) a factor of 0.68 is applied to this yield to give a corrected yield of 0.06 $\mu mol \cdot dm^{-3}$ at 200 Gy. Since the solutions of DNA were assayed at 100 $\mu mol \cdot dm^{-3}$, the ELISA technique is capable of detecting one 8,5'-cyclodeoxyadenosine moiety in 1.7 x 10^3 unchanged mononucleoside residues at the limit of detectability. Considering this limit of detectability, at least 1.2 x 19^4 Gy (2.0 x 10^4 ÷ 1.7 x 10^3 x 1000 Gy) must be delivered to cells in order to reach the lower limit of detectability of the ELISA technique.

The lower limit for detection of 8,5'-cyclodeoxyadenosine using USERIA is at 10 Gy, compared to 200 Gy for ELISA (Figure 1). Therefore, based on the aforementioned estimates of product yield, detection of 8,5'-cyclodeoxyadenosine in DNA extracted from cells irradiated to 1000 Gy was not an unreasonable objective. The failure to detect formation of this product under these conditions may be due to an overestimate of the efficiency of hydroxyl radical attack in the cellular environment, and/or to the inability to attain an assay with sufficient sensitivity.

The sensitivity of the immunochemical assays might be improved by a number of techniques. With the present assays, acid hydrolysis of the DNA to remove crossreacting moieties might possibly lead to increased sensitivity. The hydrolysis step is suitable for the 8,5'-cyclodeoxyadenosine moiety due to the acid-stability of this product. Limitations on the sensitivity of the immunochemical assays also occur as a result of limitations on the binding coefficients, in addition to nonspecific binding, of the antibodies for both the

primary and secondary antiserum. Affinity purification of antiserum is one method for selecting antibodies with the highest sensitivity. Finally, development of monoclonal antibodies or antiserum containing antibodies with increased avidity to the antigenic determinants would result in increased sensitivity.

CONCLUSIONS

Modification of the conventional ELISA by the substitution of a radiolabelled substrate (USERIA) results in a more sensitive enzyme immunoassay primarily as a result of an amplification of "signal" as compared to "noise". The limit of detectability of 8,5'-cyclodeoxyadenosine from nitrous oxide-saturated, irradiated solutions of calf thymus DNA was reduced twenty-fold with this modification. Attempts to measure 8,5'-cyclodeoxyadenosine in DNA extracted from deoxygenated, thiol-depleted, irradiated cells failed. This failure may be attributed to the inability to achieve an assay with sufficient sensitivity to overcome the lower efficiency of product formation in DNA irradiated in the cellular environment.

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CHAPTER 11

SUMMARIZING DISCUSSION

Modification of the intermediate radical centered on functionally important biomolecules (such as DNA), either by oxidizing (oxygen, electron affinic compounds) or reducing (nonprotein sulfhydryl) species, has been expounded as the molecular basis of radiosensitization and radioprotection, respectively (1-12). Evidence from the work contained within this thesis suggests that 8,5'-cyclo(deoxy)nucleoside(tide) formation may be a permanent record of the existence of a C(5')-centered radical on the nucleic acid constituent. If this is true, then this type of radiation-induced modification might serve as a molecular "probe" of the radical competition model. In an effort to develop the use of 8,5'-cyclo(deoxy)nucleoside(tide) formation as a "probe" of the radical competition model, the radiation chemistry of adenine-containing nucleic acid constituents was studied to provide the experimental basis upon which 8,5'-cyclo(deoxy)nucleoside(tide) formation can be considered unequivocal with respect to the initial site of hydroxyl radical attack (Chapters 2,4-7). Sensitive and specific assays were also developed to assess radiation-induced damage including high-performance liquid chromatography (Chapters 2-5,7), enzyme-linked immunosorbent assay (Chapters 6,7,9,10) and ultrasensitive enzyme radioimmunoassay (Chapter 10); the latter two techniques using reagent antiserum with specificity to

8,5'-cycloadenosine 5'-monophosphate.

In the absence of oxygen, irradiation of adenine-containing nucleic acid moieties leads to the formation of 8,5'-cyclo(deoxy)nucleosides(tides) (13-20). Formation of both the (R)- and (S)-epimers of 8,5'-cyclonucleotides(sides) was first demonstrated in irradiated adenosine 5'-monophosphate (5'-AMP) (16), adenosine (19) and polyadenylic acid (18,19). However, the factors governing stereoselectivity were found to be quite different in monomers and polymers (Chapter 8). At neutral pH, the (S)-epimer of 8,5'-cycloAMP predominates over that of the (R)-epimer in irradiated solutions of 5'-AMP (16) whereas, in irradiated solutions of adenosine or poly A (and probably DNA), the (R)-epimer of 8,5'-cyclo(deoxy)adenosine predominates (18,19).

The biological significance of 8,5'-cyclodeoxynucleoside formation remains an unanswered question. Formation of the C(8)-C(5') intramolecular crosslink occurs readily in native DNA irradiated in solution (Chapter 6) and, in the case of the (R)-epimer, probably involves only slight helical distortion with weakened interchain hydrogen bonding. This is in contrast to 8-hydroxynucleoside formation which involves a bulky hydroxyl substituent on the C(8) position which tends to drive the molecule into the <u>syn</u> conformation about the N-glycosylic bond resulting in disruption of interchain hydrogen bonding (21). Providing that 8,5'-cyclodeoxynucleoside formation occurs in DNA irradiated in the cellular environment, the question remains whether this lesion is recognized and repaired by the

compliment of repair enzymes within the cell. If this lesion is not repaired, what are the biological consequences of its presence in the DNA helix? Can the polymerases read through the molecular site, is the site mutagenic and can the functional integrity of the cell be maintained? The biochemical manifestations of this lesion await further investigation.

A number of lines of evidence help establish 8,5'-cyclo(deoxy)-nucleoside(tide) formation as an unequivocal probe of an initial radical event; that of hydroxyl radical-induced hydrogen atom abstraction from the C(5') carbon of the sugar moiety. Initiation of product formation by hydroxyl radical attack is confirmed by an increased yield of product in nitrous oxide- as compared to nitrogen-saturated solution (14,16) in addition to experiments where decreased yields of 8,5'-cyclonucleoside(tide) were observed as a result of $^{\cdot}OH$ scavenging by alcohols (14) and dimethyl sulfoxide (16). The concentration of oxygen required to inhibit 8,5'-cyclonucleotide formation by 50% was 0.08% pO_2 and complete inhibition occurred at 1.62% pO_2 (Chapter 4). The relative reactivity of a series of nitroaromatic radiosensitizers toward the C(5') radical intermediate in deoxygenated solutions of irradiated polyadenylic acid was assessed using standard competition kinetic analysis. Formation of the 8,5'-cycloadenosine moiety was inhibited in a way which generally increased with increasing radiosensitizer electron affinity (20). Oxidation of the radical intermediate by the radiosensitizers was the interpretation given for this observation.

Oxidation of the radical intermediate was reduced if the radiosensitizer was negatively-charged presumably because of charge repulsion with the phosphate groups (20). This evidence suggest that 8,5'-cyclo(deoxy)nucleoside(tide) formation might serve as a molecular "probe" of the radical competition model because, in the absence of oxygen, formation of this product leaves a permanent record of an initial radical event; that of hydroxyl radical-induced hydrogen-atom abstraction from the C(5') position of the sugar moiety.

Other radiation-induced molecular products, initially resulting from hydroxyl radical attack, in contrast to 8,5'-cyclo(deoxy)nucleosides(tides), are not unequivocal with respect to this initial event. Formation of 8-hydroxyadenosine 5'-monophosphate (8-hydroxyAMP) is greater in nitrous oxide-saturated solution (G = 0.14) when compared to oxygen-saturated solution (G = 0.09), but is completely inhibited in nitrogen-saturated solution (16,19, Chapter 4). However, formation of 8-hydroxyAMP in nitrogen-saturated solution can occur in the presence of > 50 $\mu mol \cdot dm^{-3}$ hydrogen peroxide (Chapter 4). These data suggest that, although formation of this product is initiated by hydroxyl radical attack presumably by addition across the C(8)-N(7) double bond, an oxidizing species is required to complete product formation. Radiation-induced adenine release exhibits a yield that is not very dependent on the presence (G = 0.46) or absence (G = 0.28) of oxygen during irradiation, an observation that is probably due to the

numerous reactions that can lead to base loss. The break in the oxygen K-curve for 8-hydroxynucleoside(tide) formation and the numerous mechanisms leading to radiolytic adenine release make these molecular lesions unsatisfactory as potential probes of the radical competition model.

Ultimately, the ability to use radiation-induced molecular modifications as "probes" of radiation chemical events involves not only an understanding of the mechanism leading to product formation, but also the development of sensitive assay techniques to detect specific products in irradiated systems. Although 8,5'-cyclo(deoxy)nucleoside formation responds in a predictable and consistent manner based on the tenents of the radical competition model in radiation chemical experiments, this product has not been detected in irradiated cells. Therefore, 8,5'-cyclodeoxynucleoside formation cannot be used as a probe of radical events in cellular systems until a method of analysis with sufficient sensitivity is developed.

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CHAPTER XII

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